PRESIDENCY UNIVERSITY

DEPARTMENT OF LIFE SCIENCES

PhD Thesis Synopsis

Identification of Molecular Markers associated with Development and clinical outcome of Gestational Diabetes Mellitus in West Bengal, India.

Name of Candidate: JAYITA BASU

Enrolment No.: SC/DOC/13-14/ 92

Registration No.: R-14RS18210019 dated 05th May 2016.

Supervisor: Dr. AMLAN GHOSH

GENETICS OF NON COMMUNICABLE DISEASES

Department of Life Sciences

Presidency University

Kolkata

Name: JAYITA BASU

Supervisor: Dr. AMLAN GHOSH

Title of Thesis: Identification of Molecular Markers associated with Development and clinical outcome of Gestational Diabetes Mellitus in West Bengal, India

PhD registration Number with date: R-14RS18210019 dated 05th May 2016.

Enrolment Number with date: SC/DOC/13-14/ 92 dated 08th May, 2014.

Place of work: GENETICS OF NON COMMUNICABLE DISEASES, Department of Life Sciences, Presidency University, Kolkata.

Residential Address: 234, Lakshmi Narayan Road, Dumdum Cantonment, Kolkata-700065.

Corresponding Address: 234, Lakshmi Narayan Road, Dumdum Cantonment, Kolkata-700065

Email: jayita.rs@presiuniv.ac.in

Phone Number: 9674074597

Jayita Basu.

(JAYITA BASU)
Signature of Research Scholar

Signature of Supervisor

Dr. Amlan Ghosh
Assistant Professor (Biological Sciences)
PRESIDENCY UNIVERSITY, KOLKATA

3rd Page

Contents

4th Page onwards

I. Abstract
II. Introduction
III. Objectives (as specified in approved 1000 word)
IV. Methodology
V. Results (Objective wise)

Objective 1:

Results with figures including legends

Objective 2, 3, 4:

Results including figure and legends

VI. Conclusion/ Discussion
VII. References
VIII. Appendix (if appropriate)

Contents

Sl. No.	Topic	Page No.
	Summary	1
1.	Introduction	2
2.	Objective of the study	5
3.	Methodology	5
3.1	Study design and recruitment of subject	5
3.2	Diagnosis of GDM	5
3.3	Samples and setting	6
3.4	Collection of samples	7
3.5	Analysis of hematological parameter	8
3.6	Total ROS measurement	8
3.7	DNA damage analysis by comet assay	8
3.8	Isolation of genomic DNA	8
3.9	Genotyping by PCR-RFLP	8
3.10	DNA sequencing	9
3.11	ELISA assay	9
3.12	Histological evaluation	10
3.13	mRNA expression analysis by quantitative real-time PCR	10
3.14	Immunohistochemical analysis	11
3.15	Statistical analysis	11
4.	Result	12
4.1.A	Study of disease prevalence and associated risk factor	12
4.1.B	Molecular basis of epidemiological link of potential risk factors	14
4.2.A	Maternal and neonatal complications	28
4.2.B	Placental histopathological abnormalities	30
4.2.C	Expression of glucose transporters in placental tissue	32
4.3.A	Association of selected genetic variance with clinical pathological parameters	35
4.3.B	Association of placental histopathological alteration and GLUT1 expression level with adverse neonatal outcome	37
5.	Discussion	39
6.	Reference	45
7.	Abbreviation	56
8.	Ethical clearance certificate	

Summary:

Gestational diabetes mellitus (GDM) is considered as health hazard with respect to short and long term adverse effect on maternal and neonatal health. On the background of fragmented knowledge, present study aimed to analyze prevalence, population specific risk factor, pathogenesis and adverse disease outcome of GDM on case control basis in a Kolkata based population of West Bengal, India. Prevalence of GDM in Kolkata was 17.2%. Older maternal age, presence of acanthosis nigricans and diabetic family history was found as major risk factors. Association of oxidative stress and related inflammation with GDM indicated incidence of insulin resistance in disease pathogenesis. Genetic predisposition seemed to play major role in development of GDM of our studied population; this was evident from significant association of genetic variants of type-2 diabetes from insulin synthesis/processing (rs7903146, rs4132670 and rs12255372 of TCF7L2 and rs2269023 of PCSK2) and secretion pathways (rs5219 of KCNJ11, rs757110 of ABCC8, rs10830963 of MTNR1B) with disease condition. As disease specific adverse neonatal outcome, macrosomia (newborn with excess birth weight) and neonatal hypoglycaemia (abnormally low blood glucose level of newborn) were highlighted in studied population. In GDM pregnancy, phenomenon of intrauterine hypoxia was evident from higher incidence of placental histological anomalies like villous edema, increased syncytial knots, chorangiosis, and thickening of basement membrane than normal one. Overexpression of GLUT1 and GLUT3 in GDM placenta was found than normal one, indicating enhanced glucose transport to fetus and was predicted as consequence of hypoxic condition. In logistic regression, villous edema, chorangiosis and placental GLUT1 were established as independent predictors for both macrosomia and neonatal hypoglycaemia and increased syncytial knots for neonatal hypoglycaemia; this indicated importance of intrauterine hypoxia and enhanced fetal glucose transport in pathogenesis of GDM associated neonatal outcome. Information of the present study might serve as background knowledge of the disease in Kolkata based population. The information might have several implications in short and long terms like designing preventive strategies for minimizing disease risk, screening strategies to identify individuals at risk before conceiving and development of suitable prognostic biomarkers to assess and modulate adverse neonatal outcome with an aim for better disease management.

Introduction

Gestational diabetes mellitus (GDM) refers to glucose intolerance recognized first during pregnancy and it resolves itself postpartum. GDM is considered as major health problem as it is associated with maternal complications during pregnancy, influences adverse neonatal outcomes and significantly increases probability of developing type-2 diabetes in GDM mother and their offspring later in life [1,2]. Prevalence of GDM is increasing alarmingly throughout the globe with overall increased incidence of diabetes [3] and Indian scenario is worse in this aspect for harboring largest number of diabetic patients all over the World [4]. Prevalence of GDM in different Indian states [2,5,6] showed wide variation (6.6–17.8%) which might be due to differences in living conditions, dietary habits, socio-economic levels etc. Therefore, multiple studies on prevalence and risk factors of GDM are warranted from different Indian region; from West Bengal, no comprehensive data in this aspect is still available to our best.

Pathogenesis of GDM is not well characterized. During pregnancy, maternal tissue becomes insulin resistant to facilitate continuous glucose transfer to fetus and when it is accompanied by insufficient secretion of insulin by pancreatic β-cells, GDM may develop[7].Therefore increased insulin resistance and its inadequate secretion seem to be major pathophysiological events in GDM. Altered physiological parameters like oxidative stress, inflammatory response etc. might have significant contribution to insulin resistance as well as its impaired secretion. Role of genetic predisposition was also presumed in impaired insulin synthesis, processing and secretion[8,9] Therefore during pregnancy, both altered physiological parameters as well as genetic predisposition seemed to play important role in development of GDM that needed to be explored for better understanding of the disease pathogenesis; however comprehensive studies in this matter is still inadequate across the globe.

GDM shared some common risk factors and patho-physiological conditions with type-2 diabetes mellitus (T2DM) [10-12]. Under hyperglycemic condition, consequences like production of disproportionate amount of free radicals [10], formation of glycosylated hemoglobin (HbA1C) as advanced glycated end product etc. significantly augment oxidative stress [11]. Oxidative stress promotes inflammatory response that interferes with insulin sensitivity and its secretion and thus contributed significantly in pathogenesis of GDM [12]. Oxidative stress induces damage to bio-molecules like lipid, DNA [13] and under inflammatory condition, hematologic-parameters like WBC, NLR (neutrophil-lymphocyte ratio) etc. show

abnormal changes [14,15] that might have potency to be biomarkers for associated patho-physiological conditions. Adipose tissue produces inflammatory mediators that increase considerably with obesity and as a result augments the risk of GDM [16]. However on the contrary, association of inflammation with gestational disorders like pre-eclamsia [17] GDM [18] etc independent of obesity was also reported. Therefore, in GDM, oxidative stress, obesity and associated inflammatory response seemed to form complex association that was not analyzed on same set of samples till date and in Indian scenario, data in this respect at preliminary level is still not available to our best.

The genetic basis of GDM is still not fully elucidated owing to absence of large scale linkage analysis or genome wide association study. From evidences like family history of T2DM as a risk factor of GDM [19] and GDM women showed higher tendency to develop T2DM in later life [20,21], it is hypothesized that genetic susceptibility to GDM would be much like that of T2DM [19]. Therefore, approaches to evaluate the role of common genetic variants of T2DM in GDM might throw light on genetic basis of the disease. During pregnancy, after development of insulin resistance, the key event leading to development of GDM seems to be inadequate secretion of insulin by β-cells of pancreas. Actually in pancreatic β-cells, insulin is synthesized as pre-proinsulin that undergoes proteolytic cleavage following additional modification to form mature insulin and ultimately secreted following a cascade of biological reaction stimulated by higher blood glucose level. However, β-cells secrete small amount of pro-insulin in addition to insulin [20], which is biologically less active compared to insulin [21].

Expression of insulin gene occurs through a complex transcriptional network of which *TCF7L2* is considered as master regulator. *TCF7L2* also seemed to regulate the processing of pro-insulin into mature insulin and the action may be exerted through regulating transcription of *PCSK1* and *PCSK2* (proprotein convertase subtilisin/kexin type-1 and 2), the genetic loci that encode PC1 and PC2 [22]. In T2DM, association of 3 intronic variants of *TCF7L2* namely rs7903146, rs12255372 and rs4132670 was reported with disease pathogenesis and allelic variation of mentioned loci was found to affect expression level of the gene [23,24]. Among genetic loci of prohormone convertases, *PCSK2* was mainly studied in T2D and one intronic variant rs2269023 showed strong link with disease risk and with diabetes associated parameters like fasting blood sugar, fasting insulin level etc [25].

For insulin secretion by pancreatic β-cells, cytosolic concentration of Ca^{2+} is needed to be raised from extracellular or intracellular source and in this processes, potassium channel (K_{ATP}Channel) encoded by *ABCC8* & *KCNJ11* and MT2 receptor encoded by *MTNR1B* play critical role [9,26]. Several genetic polymorphisms of the mentioned genes have been identified

of which association of rs5219 (*KCNJ11*), rs757110 (*ABCC8*) and rs1387153 & rs10830963 (*MTNR1B*) was reported in T2DM and allelic variation at these loci was found to be associated with impaired insulin secretion [9,26]. Therefore, with respect to insulin synthesis, processing and secretion, several candidate genes like *TCF7L2, ABCC8, KCNJ11, MTNR1B* etc. play critical role and allelic variation of the genes might predispose individuals to develop T2DM as well as GDM considering similar mode of genetic susceptibility for both the disease. Although some studies [27,19,28] explored association of few of the mentioned polymorphism in GDM, a comprehensive one taking all the potential polymorphism together in same set of samples is missing and in India, baseline data in this respect is lacking altogether.

GDM is associated with adverse neonatal outcome that seemed to be unique in comparison to other types of diabetes [29]. Placenta is an organ of short life span through which developing fetus performed all sorts of metabolic and gaseous exchange. Therefore placenta seemed to play important role in normal fetal development and any changes at histological as well as molecular level of the organ might affect such developmental process leading to various types of abnormalities of the neonates [30]. Actually placenta is a mixture of fetal (chorionic frondosum) and maternal (decidua basalis) tissues. Chorion from fetal side forms finger like projections called chorionic villi, each supplied with a branch from umbilical arteries and veins. Maternal blood directly opens into the inter-villous space and vasculo-syncytial membranes of each villous allows exchange of nutrient and gases between maternal and fetal circulation. In GDM, concentration of sugar and other nutrients increased in maternal blood leading to generation of reactive oxygen species (ROS), related inflammatory mediators etc. In response to such stressed environment, placental changes at histological and molecular level especially differential expression of nutrient transporter and stress response molecule were predicted [31]; chorionic villi of placenta as remain in direct contact of maternal blood at the site of exchange between maternal and fetal circulation, seemed to be susceptible site for such changes. Histopathological studies of placenta in GDM women are relatively rare. Although in few studies [30,32,33], histological abnormalities like syncytial knot, chorangiosis, nucleated fetal red blood cell (NFRBC), calcification etc. were found more frequently in GDM placenta compared to non-GDM one, clinical significance of such abnormalities with adverse neonatal outcome remained unexplored. Among molecular markers, expression of glucose transporters particularly at the site of chorionic villi seemed to be an important phenomenon in GDM women. In response to high sugar level in maternal blood, expression of glucose transporters may be up-regulated leading to enhanced glucose transport into the

fetus that might contribute significantly to neonatal complications like hyper-insulinimia, fetal hypoglycemia etc. Expression of different glucose transporters like GLUT1, GLUT3, GLUT4, GLUT5, GLUT8, GLUT9 and GLUT12 was detected in human placenta [34,35,36,37, 38,39] and over-expression of some selected candidates in diabetic pregnancy were reported. However, except only one study [38] with very few number of samples, expression status of GLUTs in GDM placenta was not studied till now and furthermore association of this phenomenon with adverse neonatal outcome remains unexplored altogether.

On the basis of aforesaid backdrop present hospital based case control study aimed to determine prevalence of GDM and analyze associated risk factors in patients from Kolkata, West Bengal; association of potential molecular markers (genetic and non-genetic) that might influence insulin synthesis/processing, secretion and sensitivity was analyzed with disease pathogenesis; finally alterations of placental tissue at histological and molecular level was studied to explore its putative association with adverse neonatal outcome.

2. Objective of the study

1. Identification of molecular markers associated with development of gestational diabetes mellitus.
2. Analysis of expression profile of selected genes in placental tissue to explore the molecular basis of disease outcome.
3. Clinicopathological association of disease specific molecular markers.

3. Methodology

3.1. Study design and recruitment of subject:

It was a hospital-based case-control study conducted in Department of Gynecology and obstetrics of Institute of Postgraduate Medical Education and Research (IPGME&R). Studied protocol was approved by institutional ethical committee of respective hospital and consent in written form was taken from participating individuals. Pregnant women of 24th–28th weeks of gestation who visited the hospital during study period [January 2016- March 2017] were included in our study. Patients with previous history of diabetes mellitus or any chronic disorder were excluded.

3.2. Diagnosis of GDM

GDM diagnosis of pregnant women was done at 24th–28th weeks of gestation by oral glucose tolerance test (OGTT) according to the Diabetes in Pregnancy Study Group India (DIPSI) guideline [40]. Briefly, 75-gram glucose was given to participants after overnight fasting (8–12

h) and plasma glucose level from venous blood was detected after 2hrs by GOD-POD method. Study participants having blood glucose level<120mg/dl in OGTT were considered as non-GDM and with 120-139 and ≥140mg/dl as GI (glucose intolerance) and GDM respectively.

3.3. Samples and setting:

Total 735 eligible candidates participated initially that sounds good for prevalence study with respect to minimum number (n=589) of individuals needed for such purpose (95% confidence interval and 3% precision) considering overall prevalence rate (16.55%) of the disease in India [40]. GDM (n=127) women were considered as case, non-GDM (n=596) as control and GI (n=12) individuals were excluded for further analyses associated with the study.

For study of disease pathogenesis, 5-6 ml blood sample was taken from the willing individuals in fasted condition and to explore molecular basis of disease associated fetal/neonatal outcome, part of placental (term) tissue was collected in addition after parturition. From initially eligible participant, case and control individuals were selected primarily based on matched socio-demographic characters and then number of participants in different analyses of the study was allotted based on willingness as well as availability of sufficient amount of samples. A summary of the study plan including usage of blood and tissue samples in different analyses was schematically shown in Figure-1.

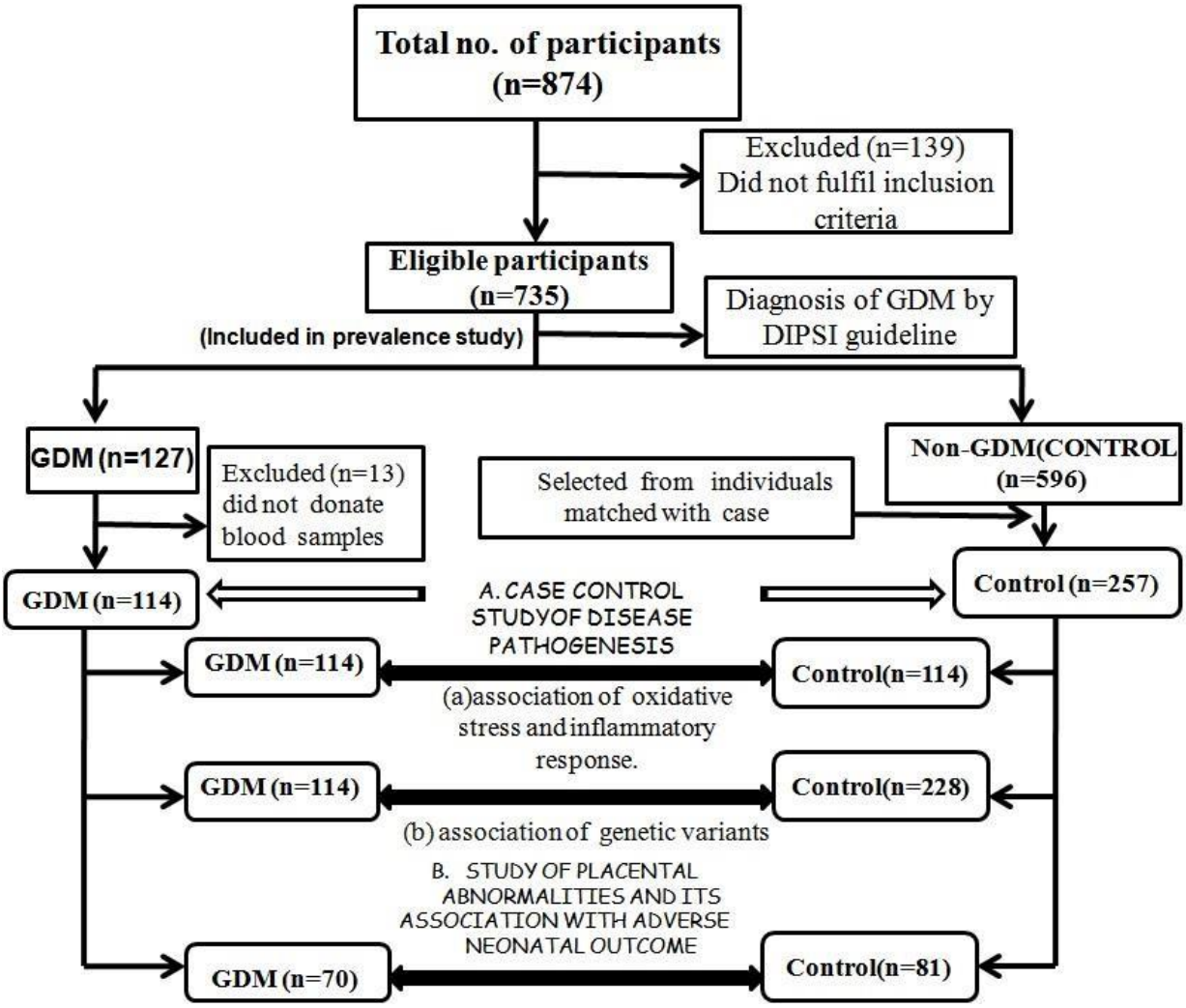
Total no. of participants (n=874)
Excluded (n=139) Did not fulfil inclusion criteria
Eligible participants (n=735)
(Included in prevalence study)
Diagnosis of GDM by DIPSI guideline
GDM (n=127)
Excluded (n=13) did not donate blood samples
Selected from individuals matched with case
Non-GDM(CONTROL (n=596)
GDM (n=114)
A. CASE CONTROL STUDYOF DISEASE PATHOGENESIS
Control (n=257)
GDM (n=114)
Control(n=114)
(a)association of oxidative stress and inflammatory response.
GDM (n=114)
Control(n=228)
(b) association of genetic variants
B. STUDY OF PLACENTAL ABNORMALITIES AND ITS ASSOCIATION WITH ADVERSE NEONATAL OUTCOME
GDM (n=70)
Control(n=81)

Fig 1: Summary of the overall study plan. GDM, gestational diabetes mellitus; DIPSI, Diabetes in Pregnancy Study Group India; n, total number of samples.

3.4. Collection of samples:

a) **Blood sample collection:** 5-6 ml of blood was collected from selected individuals in fasting condition. Depending upon the available amount, each sample was divided into maximum three aliquots. An aliquot of blood was used for DNA extraction and its serum part was stored at -20°C for ELISA assay; from another two, hematological analysis was done and lymphocytes were separated using histopaque®-1077 (Sigma) for ROS measurement and comet assay.

b) Placental tissue collection: Placental tissue samples were collected only from case (n=70) and control individuals (n=81) who were successfully followed-up till the time of delivery. Immediately after delivery, term placenta was obtained and from disc region approximately

positioning at midpoint between insertion site of umbilical cord and placental margin, multiple tissue samples of around 1cm^{3}size were collected. Part of tissue sample was directly taken in TRIZOL for mRNA isolation and other fixed in formalin (10%) followed by paraffin embedding for histological and immunohistochemical analyses. Neonatal detail was documented from respective unit of the hospital.

3.5. Analysis of hematological parameters:

Complete blood count (CBC) and measurement of glycosylated hemoglobin (HbA1C) was done from whole blood samples. The analyses were done in pathology department of IPGME&R by Sysmex XS-800i automated hematology analyzer and high performance liquid Chromatography (HPLC) respectively.

3.6. Total ROS Measurement:

Total ROS level was measured in lymphocytes isolated from the blood samples, using DCFH-DA (2,7-dichlorofluorescein diacetate; Sigma, USA) by standard protocol. Spectro- fluorimeter (CARY Eclipse Varian) was used to measure fluorescent intensity at excitation wave length of 475nm and emission wavelength of 523 nm and data was presented as AFU (arbitrary fluorescence units)/10^4 cells [41].

3.7. DNA damage analysis by Comet assay

DNA damage was measured in peripheral blood mononuclear cells and alkaline comet assay was used for this purpose. In brief, a mixture of cell suspension and 2% low melting point agarose was prepared in 1:1 proportion and immediately placed on a glass slide previously coated with 1% agarose. When gel was solidified, slides were incubated with cold lysis buffer at 4^0Cfor 1 hour. Slides were then incubated with alkaline electrophoresis buffer at pH-13 that allowed DNA to unwind for expression of alkali-labile damage. Next electrophoresis was done followed by neutralization of the slides, staining with EtBr and mounting in glycerol. Finally, slides were visualized under fluorescent microscope (Leica DM4000 B, Germany) and open comet (computerized image analysis system) tool was used to measure different comet parameters.

3.8. Isolation of genomic DNA: For isolation of genomic DNA from blood samples, phenol chloroform method was employed following standard protocol [42].

3.9. Genotyping by PCR-RFLP: PCR product with selected polymorphism was restriction fragmented using specific enzymes followed by agarose gel electrophoresis and genotypes for each polymorphism were documented from band pattern of restriction fragments

visualized on gel documentation system (Universal Hood III, Bio-Rad). Primer sequences and restriction enzymes used in the analysis, listed in Table-1.

Table 1: List of Primers and Restriction Enzymes Used for SNPs genotyping:

GENE	SNPs	PRIMERS	AMPLICON SIZE(bp)	Tm (^{0}C)	ENZYME
TCF7L2	rs7903146	5′-ACAATTAGAGAGCTAAGCACTTTTTAGGTA-3′ 5′- GTGAAGTGCCCAAGCTTCTC-3′	188bp	55^{0}C	RSal
	rs12255372	5′-CTGGAAACTAAGGCGTGAGG-3′ 5′- GGGTCGATG TTGTTGAGCTT-3′	244bp	56^{0}C	MLUC1
	rs4132670	5′-GCTCAGCAAGAGATCAAGTGG-3′ 5′-TTGCTATCTGCCTGCCTTTT-3′	258bp	55^{0}C	BsrD1
PCSK2	rs2269023	5′-CAGGCTGATTAATTCCCAGTGCTGTTAGCAG-3‟ 5′-AGGTGTCCATGGTCCTTACG-3′	171bp	58°C	Tse-1
KCNJ11	rs5219	5′-TCCCGGATGTTCTTGTGG-3′ 5′-GACTCTGCAGTGAGGCCCTA-3′	235bp	55^{0}C	BanII
ABCC8	rs757110	5′-GGGAAGAGTCCAAGGAGGAG-3′ 5′-CAGGAGACTGCGATGTCTGA-3′	344bp	56^{0}C	MwoI
MTNR1B	rs1387153	5′-ACCATTCTCAGTGGTCCTTACT-3′ 5′-GGGCCTAAGAGCCTCCATTT-3′	169bp	53^{0}C	BstN1
	rs10830963	5‟GGCCCCCAGTCATGCTAAGAATTCACACCATAT3‟ 5‟TTGGAAGAGAGGAGGCAAGG3‟	155bp	59^{0}C	NdeI

Tm, Primer Melting Temperature; ^{0}C, degree Celsius; bp, base pairs

3.10. DNA sequencing: For validation, samples of representative genotypes were subjected to direct sequencing using specific primer set mentioned in Table-1. In each sample, both strands were subjected to sequencing by ABI PRISMTM BD Terminator Cycle Sequencing kit (PE Applied Biosystems, Foster City, CA, USA) and electrophoresed in 3100-Avant Genetic Analyzer (PE Applied BiosystemsInc,USA). The electropherograms obtained were subjected to sequencing analysis software (version 1.01, Applied Biosystem); ultimately forward and reverse complement sequences were aligned with corresponding reference sequences (ensemble.org) by Clustal W multiple alignment software program (European Bio Informatics Institute) to detect specific sequence variations of the respective polymorphism.

3.11. Biochemical measurement: Estimation of fasting plasma glucose was done using GOD-POD method on a RXIMola automated analyzer, Randox, UK. Proinsulin, Insulin and C-peptide were measured from fasting serum sample of 59 GDM women and equal number of non-GDM control. Analyses were done by Enzyme-linked immune-sorbent (ELISA) assay using commercially available kit. Sandwich ELISA method was employed for detection of pro-insulin

and insulin using Human PI(Proinsulin) (Fine Test; catalogue no.EH0388) and Human Insulin (INS) (Krishgen BioSystems; Item code, K12-0011) ELISA kits; C-peptide was assessed by competitive ELISA using RayBio® C-Peptide Enzyme Immunoassay Kit (cat no. EIA-CPE, EIAM-CPE, EIAR-CPE) according to manufacturer's instruction. For evaluating insulin resistance/sensitivity, homeostasis model assessment of insulin resistance (HOMA-IR) and quantitative insulin sensitivity check index (QUICKI) was calculated using standard formula; HOMA-IR= fasting insulin (μU/ml)× fasting glucose (mmol/l)/22.5 and QUICKI=1/log (fasting glucose)+ log (fasting insulin).[43] Evaluation of β-cell function was done by homeostasis model assessment of β-cell function (HOMA-β) and was calculated as follows: HOMA- β = (20× fasting insulin, μU/ml) / (fasting glucose, mmol/L-3.5).[43]

3.11. Histological evaluation:

Total 70 GDM and 81 controls individual were included in the analysis based on availability of term placental samples. Briefly 3-5μm paraffin sections were subjected to haematoxylin- eosin staining for histological evaluation. Placental histological evaluation (abnormalities) was done by reputed pathologist under high power light microscope according to standard procedure.

3.12. mRNA expression analysis by quantitative real-time PCR (qRT-PCR):

mRNA expression analysis of glucose transporters (GLUTs) was done in human term placental tissue of GDM and non-GDM categories (n=50 for each) by reverse transcriptase PCR (RT-PCR); GAPDH was used as internal control [44]. Primer sequences used in the analysis were given in table-2. From tissue samples, total RNA was extracted using TRIzol reagent (Invitrogen, USA) and was reverse transcribed to cDNA by high-capacity cDNA reverse transcription kit (Thermo Fisher Scientific) according to the manufacturer's protocols. Conventional PCR followed by agarose gel electrophoresis was done to check the amplicon of correct size. Real time quantification of mRNA of selected genes was done by BioRad CFX96 using iTaq Universal SYBR Green Supermix (BioRad). Threshold cycle (Ct) of candidate GLUTs was normalized against Ct value of GAPDH in same sample to calculate dCt. Comparative threshold cycles (ddCt) in GDM cases were calculated taking the mean dCt value of non-GDM control as calibrator. Finally relative expression of each candidate gene (GLUTs) was calculated by the formula 2^{-ddCt} [45].

Table 2: List of Primers used for mRNA expression analysis

TARGET GENE	PRIMERS	LOCATION	AMPLICON SIZE(bp)
GLUT1	5′-ACCATTGGCTCCGGTATCG-3′ 5′-CCAGGACCCACTTCAAAGAA-3′	Exon 7-9	224
GLUT3	5′-GGAAAGGGCAGGAAGAAGGA-3′ 5′-GCCTGGTCCAATTTCAAAGA-3′	Exon 8-9	271
GLUT4	5'-CTTCGAGACAGCAGGGGTAG-3' 5′-CAGAGCCACAGTCATCAGGA-3′	Exon 8-9	175
GLUT8	5'-TGCTGCTTCTCATGTGCTTC-3' 5'- TAGAACATGACGGCGTTGAC-3'	Exon 5-6	250
GLUT9	5'-AGGACCGAGGAGAAGATGAAG-3' 5'-TGCCTTCTTTCCCATGACTC-3'	Exon 3-5	187
GLUT12	**5'-** CTAGCATGAACTGGGGCATC-3' **5'-** ACAACAAAAAGCAGGGATGC-3'	Exon 5-6	250
GAPDH	5'-AAGGCTGTGGGCAAGGTC-3' 5'- CTGCTTCACCACCTTCTTGA-3'	Exon 8	150

3.13. Immunohistochemical analysis:

Immunohistochemical analysis of GLUT1 and GLUT3 proteins was done in paraffin-mounted sections of placental tissue from GDM (n=70) and non-GDM control (n=81) according to standard protocol. Rabbit monoclonal IgG, ab-115730 (abcam) for GLUT1 and mouse monoclonal IgG1, SC-74497 (Santa Cruz Biotechnology) for GLUT3 as primary and HRP conjugated goat anti-rabbit (SC-2004) and goat anti-mouse (SC-2005) secondary antibodies were used according to manufactures protocol. Slides were developed using chromogen (3-3'diaminobenzidine, Santa Cruz), counter stained with hematoxylin and photographed in bright-field microscope (Leica DM1000, Germany). In placenta, chorionic villi were the structure of interest and GLUT1 & GLUT3 proteins in syncytio-trophoblast layer and fetal capillaries of each villous was considered for scoring based on previous report [46,35]. Around 200 villi were examined in each sample and both percentage of positive villi (1-20=1, 20-50=2, 50-80=3 and >80=4) and staining intensity (1-weak, 2-moderate, 3-strong) was scored following standard protocol [47] with some modification. Ultimately by combination of two scores, a value of 1-12 was generated on final evaluation of expression.

3.14. Statistical analysis:

Descriptive statistics was used to represent socio-demographic characters, risk factors, maternal complication and adverse neonatal outcome of study subjects. Data of discrete variable was analyzed by Fisher's exact test& chi-square test for comparison. Data of

continuous variable were tested for normality by Shapiro-Wilk method and Mann Whitney-U test was done accordingly. Association of different parameters with disease consequenc was determined by Spearman's correlation test. Linear regression analysis of univariate and multivariate types were done to evaluate role of different parameters in disease pathogenesis after adjustment of cofounders. Logistic regression analysis was done to predict impact of risk factors, histological and molecular abnormalities on disease development as well as unfavorable disease outcome (neonatal). All statistical analyses were two sided with $p<0.05$ considered as significant and were done using Graph Pad PRISM (version-7, 2016) and SPSS 17 for Windows (Chicago, USA).

Results

Objective 1: Identification of molecular markers associated with development of gestational diabetes mellitus

A. Study of disease prevalence and associated risk factors

GDM was detected in 17.2% (127/735) of participants on hospital basis. Among risk factors, univariate analysis showed significant association of maternal age ($p=0.003$), pre- gestational BMI (body mass index) ($p=0.006$), acanthosis nigricans ($p<0.0001$) and family history of diabetes (FHD) ($p<0.0001$) with GDM (Table-3a). In multivariate analysis, except pre-gestational BMI, association of maternal age (OR: 1.53; $p=0.044$), acanthosis nigricans (OR: 3.61; $p<0.0001$), and FHD (OR: 2.034; $p=0.001$) remained significant (Table-3b) and thus seemed to be potential risk factors of disease in the studied population.

Table-3a: Socio-demographic characteristics of study population

		GDM (n=127)	Non GDM (n=588)	P value
Age (year)	Mean ± SD	25.78 ± 4.89	24.34 ± 4.32	0.003*
Pre-gestational BMI (Kg/m2)	Mean ± SD	25.69 ± 4.06	22.73 ± 3.98	0.006*
Family history of diabetes	Yes	57 (45%)	130 (22%)	<0.0001*
	No	70 (55%)	458 (78%)	
Acanthosis nigricans	Yes	79 (62%)	158 (27%)	<0.0001*
	No	48 (38%)	430 (73%)	
Educational level	Illiterate	18 (14%)	50 (9%)	0.23
	10^{th} Standard	78 (61%)	394 (67%)	
	Graduation or above	31 (25%)	144 (24%)	
Socioeconomic Status	Upper	5 (4%)	35 (6%)	0.81
	Upper middle	19 (15%)	112 (19%)	
	Lower middle	52 (41%)	212 (36%)	
	Upper lower	50 (39%)	217 (37%)	
	Lower	1 (1%)	12 (2%)	

GDM, Gestational diabetes mellitus; BMI, Body mass index; * p value significant.

Table-3b: Multivariate logistic regression analysis of risk factors associated with GDM

		Number (%)	OR	95% CI for OR	*p*-value
Age	≥25 Years	261 (37%)	1.526	1.012-2.302	**0.044***
	<25 Years	454 (63%)			
Acanthosis nigricans	Yes	237 (33%)	3.609	2.240-5.815	**<0.0001***
	No	478 (67%)			
Family history of diabetes	Yes	187 (26%)	2.035	1.323-3.130	**0.001***
	No	528 (74%)			
BMI (Pre-gestational)	≥25kg/m^2	211 (30%)	1.088	0.673-1.759	0.731
	<25 kg/m2	504 (70%)			

OR, Odds ratio; CI, Confidence interval; * p-value significant.

B. Molecular basis of epidemiological link of potential risk factors

Among potential risk factors, acanthosis nigricans indicated morphological changes in skin complexion under influence of insulin resistance; oxidative stress, related inflammation etc. were known to play important role in this phenomenon. On the other hand, individual with FHD seems to inherit genetic defect mostly associated with insulin synthesis, processing and secretion pathway. Thus in this objective, to explore molecular basis of disease pathogenesis (mechanism of disease development), the study plan is considered under two broad heads:

a. **<u>Association of oxidative stress and related inflammation in disease pathogenesis</u>**

The analysis was done in 114 GDM and equal number of control subjects from the study participants depending upon availability of samples and other criteria. The study plan in detail was summarized in Figure-2.

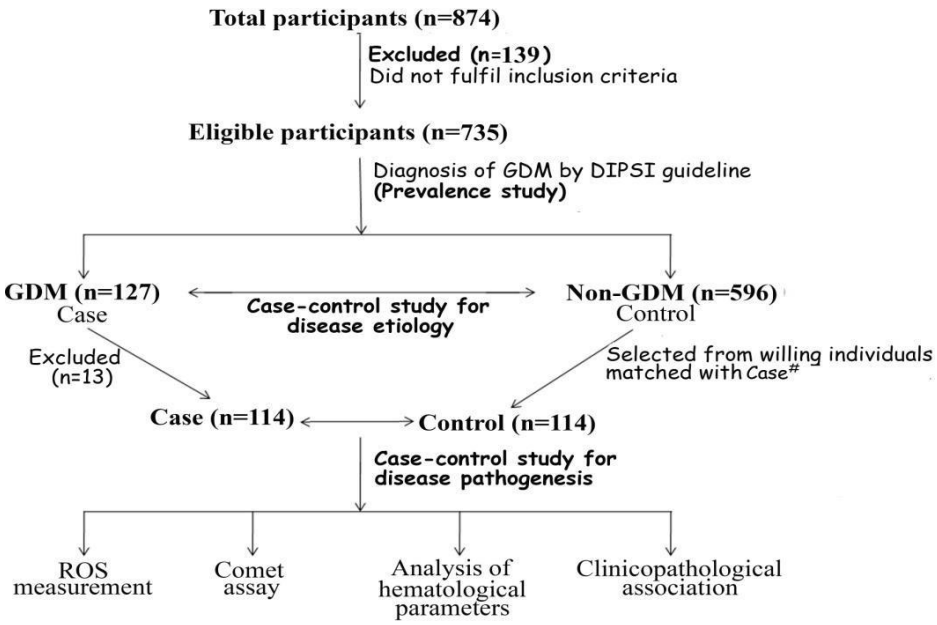

Fig 2: Summary of case and control subjects participated in pathogenesis study. GDM, gestational diabetes mellitus; DIPSI, Diabetes in Pregnancy Study Group India; ROS, reactive oxygen species; n, total number of samples; #matching between case and control with respect to maternal age, family history of diabetes, acanthosis nigricans, educational level and socioeconomic status.

i. ROS & hematological parameter in GDM and control subjects

Oxidative stress was measured as total reactive oxygen species (ROS) in blood and it was significant higher ($p<0.0001$) in GDM compared to non-GDM control. Among hematological parameters, glycosylated hemoglobin (HbA1C) was considered as disease consequence and white blood cell (WBC), red blood cell (RBC), neutrophil to lymphocyte ratio (NLR), mean platelet volume (MPV) etc. as the inflammatory parameters that were found significantly higher in GDM compared to control subject (Table-4).

Table-4: Comparison of total ROS & haematological parameters between GDM & Control subjects

U test	GDM (Mean±SD)	CONTROL(Mean±SD)	*p*-value
ROS (AFU)	186.1±43.2	104.6±27.7	<0.0001*
HbA1C (%)	6.00 ± 0.66	4.66±0.24	<0.0001*
WBC (10^9/L)	11.95±2.51	9.09±1.90	<0.0001*
RBC (10^{12}/L)	4.14±0.42	3.87±0.39	0.0001*
PLT (10^9/L)	230±73.8	249±77.3	0.0647
PDW (fL)	17.27±3.05	16.69±2.78	0.058
NLR	2.87±0.91	1.79±0.51	<0.0001*
MPV (fL)	11.9 ± 1.49	10.42±0.7	<0.0001*
MCV (fL)	87.9±6.9	86.8±7.9	0.1008
HGB (g/dL)	11.35±1.22	10.89±1.15	0.001*
HCT (%)	34.34±5.17	32.63±3.22	<0.0001*
MCH (pg)	28.15±3.16	28.09±3.26	0.81
MCHC (g/dL)	31.80±1.71	32.20±1.58	0.063

SD, standard deviation; ROS, reactive oxygen species; AFU, arbitary fluorescent unit; WBC, white blood cell; RBC, red blood cell; PLT, platelets; PDW, red blood cells volume distribution width; NLR, neutrophil–lymphocyte ratio; MPV, mean platelet volume; MCV, mean cell volume; HGB, hemoglobin; HCT, hematocrit; MCH, mean cell hemoglobin; MCHC, mean corpuscular hemoglobin; * p value significant.

ii. Assessment of DNA damage in mononuclear blood cells

Analysis of DNA damage by comet assay was done to assess the impact of oxidative stress on cellular components. Comet tails observed in the study were categorized into 4 distinct types based on extent of DNA damage (Figure-3A); type-1 (low DNA damage), type-2 (moderate DNA damage) and type-3&4 (severe DNA damage). Frequency of Comet tails

with moderate and severe DNA damage were significantly higher ($p<0.001$) in GDM women compared to that of control (Figure-3Ba). The percentage (%) of DNA in comet tail was also significantly higher in GDM compared to that of non-GDM control (Figure-3Bb).

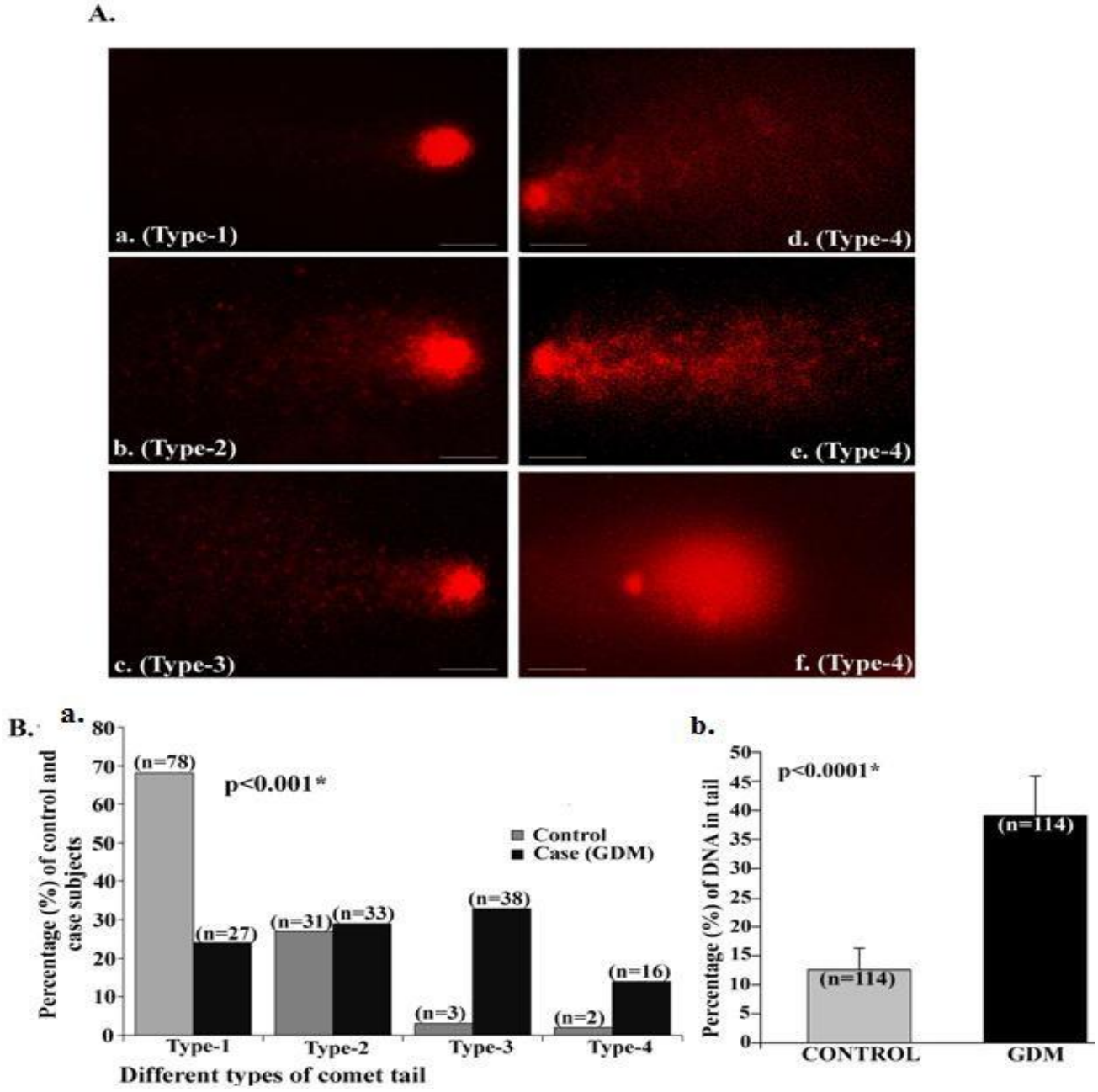

Fig 3: (A) Evaluation of DNA damage by comet assay; four types of comet tail with increasing DNA damage from type-1 to 4: type-1, very short or negligible tail; type-2, long tail but majority of DNA in head; type-3, very long tail with evenly distributed DNA; type-4, very long tail (may be balloon shaped) with majority of the DNA. Original magnification, X40; scale bar, 40µm. (Ba) Pattern distribution of comet tail types among GDM and control subjects. (Bb). Histograms showing percentage (%) of DNA in comet tail in GDM and control subjects *p-value significant.

iii. Association of HbA1C with oxidative stress and hematological parameter

To assess the impact of oxidative stress and related inflammatory response in disease pathogenesis, correlation of these parameters with HbA1C, a disease consequence, was determined (Figure-4). In GDM women, total ROS (r=0.720, p<0.0001) and comet tail DNA% (r=0.696, p<0.0001) showed strong and significantly positive correlation with HbA1C. Among potential inflammatory parameters, association of WBC (r=0.689, p<0.0001), NLR (r=0.672, p<0.0001), MPV (r=0.336, p=0.0002), RBC (r=0.256, p<0.005), and HCT (r=0.193, p=0.039) with HbA1C remained significant, although association strength seemed to be modest to week.

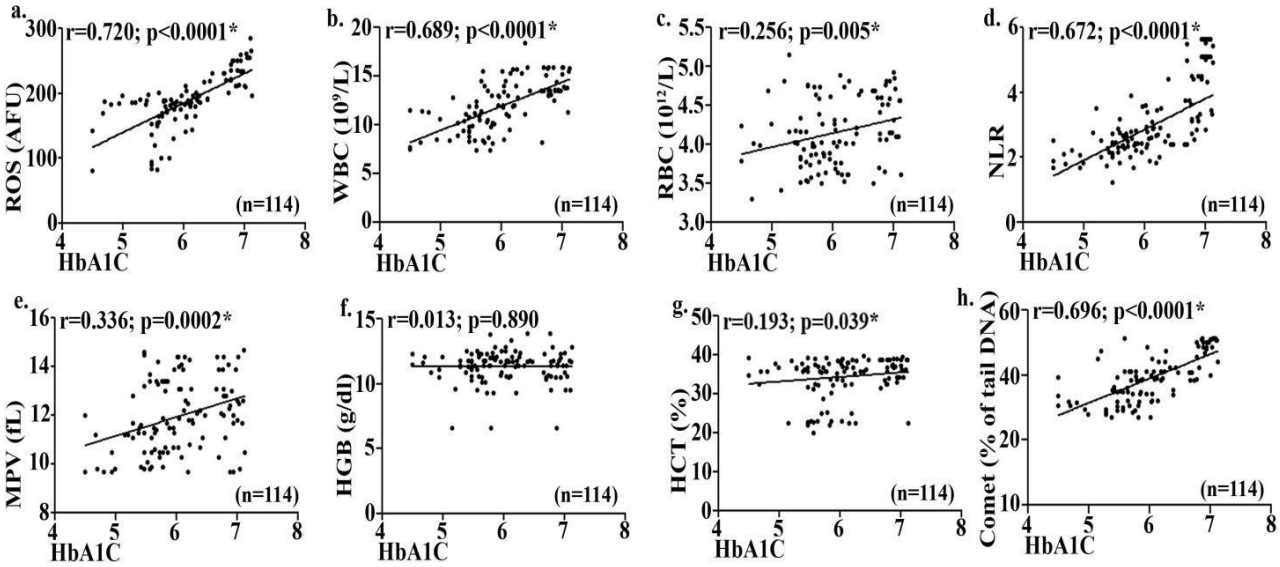

Fig 4: (a) Interrelationship of glycosylated haemoglobin HbA1C with total reactive oxygen species (ROS) a, haematological parameters (b-g) and comet tail DNA % (h). WBC, white blood cell; RBC, red blood cell; NLR, neutrophil–lymphocyte ratio; MPV, mean platelet volume; HGB, haemoglobin; HCT, haematocrit. *p-value significant. AFU, arbitrary fluorescent unit; p-value significant.

iv. Linear regression analysis

Parameters of oxidative stress, DNA damage and inflammation were selected from correlation test and linear regression analysis was done for further confirmation of their association in disease pathogenesis. In univariate analysis total ROS, comet tail DNA %, WBC,RBC, NLR, and MPV showed significant association with HbA1C (dependent variable) after adjustment for BMI (**Table 5a**). Finally in multivariate analysis, ROS (B=0.120, p<0.0001), WBC (B=0.116, p=0.003), comet tail DNA %

(B=0.106, p=0.043), MPV (B=0.218, p<0.0001) and NLR (B=0.110, p<0.0001) were predicted as independent determinants of disease condition (**Table 5b**).

Table-5a: Linear regression analysis of independent variables against HbA1C (Glycosylated hemoglobin) in GDM women after adjusting for BMI.

	Ln HbA1C			
	B[#] (95%CI)	**t**	**β[$]**	***p*-value**
ROS	0.255 (0.195-0.316)	8.388	0.621	<0.0001*
Comet[†]	0.405 (0.319-0.491)	9.335	0.665	<0.0001*
WBC	0.328 (0.254-0.403)	8.699	0.635	<0.0001*
RBC	0.276 (0.076-0.476)	2.732	0.252	0.007*
HCT	0.075 (-0.048-0.197)	1.211	0.114	0.229
NLR	0.250 (0.198-0.302)	9.489	0.669	<0.0001*
MPV	0.315 (0.158–0.473)	3.964	0.351	<0.0001*

Table-5b: Multivariate linear regression analysis of selected independent variables against HbA1C (Glycosylated hemoglobin) in GDM women.

	Ln HbA1C			
	B[#] (95%CI)	**t**	**Beta[$]**	***p*-value**
ROS	0.120 (0.056-0.184)	3.733	0.279	<0.0001*
Comet[†]	0.106 (0.003-0.209)	2.044	0.174	0.043*
WBC	0.116 (0.039-0.193)	2.996	0.218	0.003*
RBC	-0.089 (-0.228-0.050)	-1.272	-0.081	0.206
NLR	0.110 (0.050-0.170)	3.638	0.294	<0.0001*
MPV	0.218 (0.116 – 0.319)	4.257	0.239	<0.0001*

B[#,] Non-standardized coefficient; **β[$]**, Standardized coefficient; BMI, body mass index.ROS, reactive oxygen species; WBC, white blood cell; RBC, red blood cell; NLR, neutrophil–lymphocyte ratio; HCT, hematocrit; OGTT, oral glucose tolerance test; [†]Percentage of DNA in comet tail;* p-value significant.

b. Association of common genetic variants of T2DM in pathogenesis of GDM:

Total 114 GDM cases and 228 non-GDM controls were selected for the analysis from initial study participants depending upon their willingness, availability of samples and for keeping parity among case and control with respect to certain criteria (Table-6). Among samples, 114 GDM cases and 114 controls constituted the common sample pool used for studying disease pathogenesis.

Table 6: Demographic characters of study subjects

		GDM (n=114)	Non GDM (n=228)	P value
Age (year)#		25.76 ± 5.03	24.77 ± 4.44	0.1134
BMI (Kg/m2)#		24.73 ± 3.45	23.81 ± 2.95	0.1347
Fasting blood sugar(mg/dl)#		96.21±2.26	82±5.6	<0.0001*
2hour-PPBG(mg/dl)#		166.32 ± 35.34	105.56 ± 13.33	<0.0001*
Family history of diabetes	Yes	52(45.6%)	81(35.5%)	0.08
	No	62(54.4%)	147(64.5%)	
Acanthosis nigricans	Yes	71(62.3%)	116(50.9%)	0.051
	No	43(37.7%)	112(49.1%)	
Educational level	Illiterate	15(13.2%)	26(11.4%)	0.85
	10th Standard	70(61.4%)	139(61%)	
	Graduation or above	29(25.4%)	63(27.6%)	
Socioeconomic Status	Upper	4(3.5%)	12(5.3%)	0.81
	Upper middle	19(16.7%)	42(18.4%)	
	Lower middle	46(40.4%)	87(38.2%)	
	Upper lower	44(38.6%)	82(35.9%)	
	Lower	1(0.8%)	5(2.2%)	

FPG, Fasting plasma glucose; PPBG, Postprandial Blood Glucose; *p value is significant

i. Genetic variants associated with insulin synthesis and processing pathway

Table 7: List of selected SNPs:

GENE	SNPs	LOCATION	MAJOR/MINOR ALLELE	MAF(1000 GENOME)
TCF7L2	rs7903146	INTRON 3	C/T	0.23
	rs12255372	INTRIN 4	G/T	0.21
	rs4132670	INTRON 3	G/T	0.34
PCSK2	rs2269023	INTRON 8	C/T	0.39
KCNJ11	rs5219	EXON 1	C/T	0.26
ABCC8	rs757110	EXON 33	T/G	0.27
MTNR1B	rs1387153	5′ REGION	C/T	0.35
	rs10830963	INTRON 1	C/G	0.26

Three single nucleotide polymorphisms rs7903146, rs4132670 and rs12255372 from intronic region of TCF7L2 and one from PCSK2 (Table-7) were selected for the study. The genotypes of each variant (SNP/ single nucleotide polymorphism) were readily detected in

our studied population (Figure-5) and the genotypic frequencies did not show significant deviation (p>0.05) from Hardy-Weinberg equilibrium in studied subjects.

Variants of TCF7L2: For all the three studied variants of *TCF7L2*, minor allele was T. Allelic and genotypic frequencies were presented in table-8A and 8B. In rs12255372 and rs4132670, frequency of T allele was significantly higher (p=0.0008 and p=0.002 respectively) in GDM cases than non-GDM control (Table-8A). Therefore 'T' seemed to be the risk allele and in rs12255372 and rs4132670 it was associated with 2.67 times (95%CI=1.51-4.72) and 2.26 times (95%CI=1.32-3.87) higher risk for developing GDM in the studied population. In rs7903146, although T allele was more frequent in case individuals compared to control, the difference did not reach to statistical significance (34% versus 28%, p=0.25); however genotypic distribution of the studied SNPs showed significant difference between GDM and non-GDM subjects (Table-8b). In logistic regression analysis with rs12255372, GT genotype with TT assuming dominant genetic model showed about two fold increased disease risk than GG (OR=1.99, 95%CI=1.22-3.28, p=0.006) and TT assuming recessive genetic model showed about four fold increased risk than GG&GT (OR=4.47, 95%CI=1.29-15.49, p=0.018) after adjustment of potential confounders (Table-9). Similarly rs4132670 also showed two fold and four fold increased risk in dominant (CT+TT versus CC, OR=2.31, 95%CI=1.44-3.71, p=0.001) and recessive genetic models (TT versus CC+CT, OR=3.91, 95%CI=1.47-10.44, p=0.006) respectively. However in rs7903146, association of genotypes with increased disease risk remained significant in recessive model only (TT versus CC+CT, OR=2.85, 95%CI= 1.26-6.39, p=0.011), but not in dominant one.

Variant of *PCSK2*: In rs2269023, comparatively higher frequency of T allele in case than in control individuals (49% versus 38.2%; p=0.063) indicated it as the probable risk allele in studied population (Table-8a). Significant difference was found (p=0.018) in genotypic distribution of the SNP between case and control subjects. Moreover the SNP also showed significant association with higher GDM risk in dominant (CC versus CT+TT, OR=1.8, 95%CI=1.08-2.97, p=0.02) and recessive (CC+CT versus TT, OR=1.86, 95%CI=1.04-3.34, p=0.03) genetic models (Table-8B).

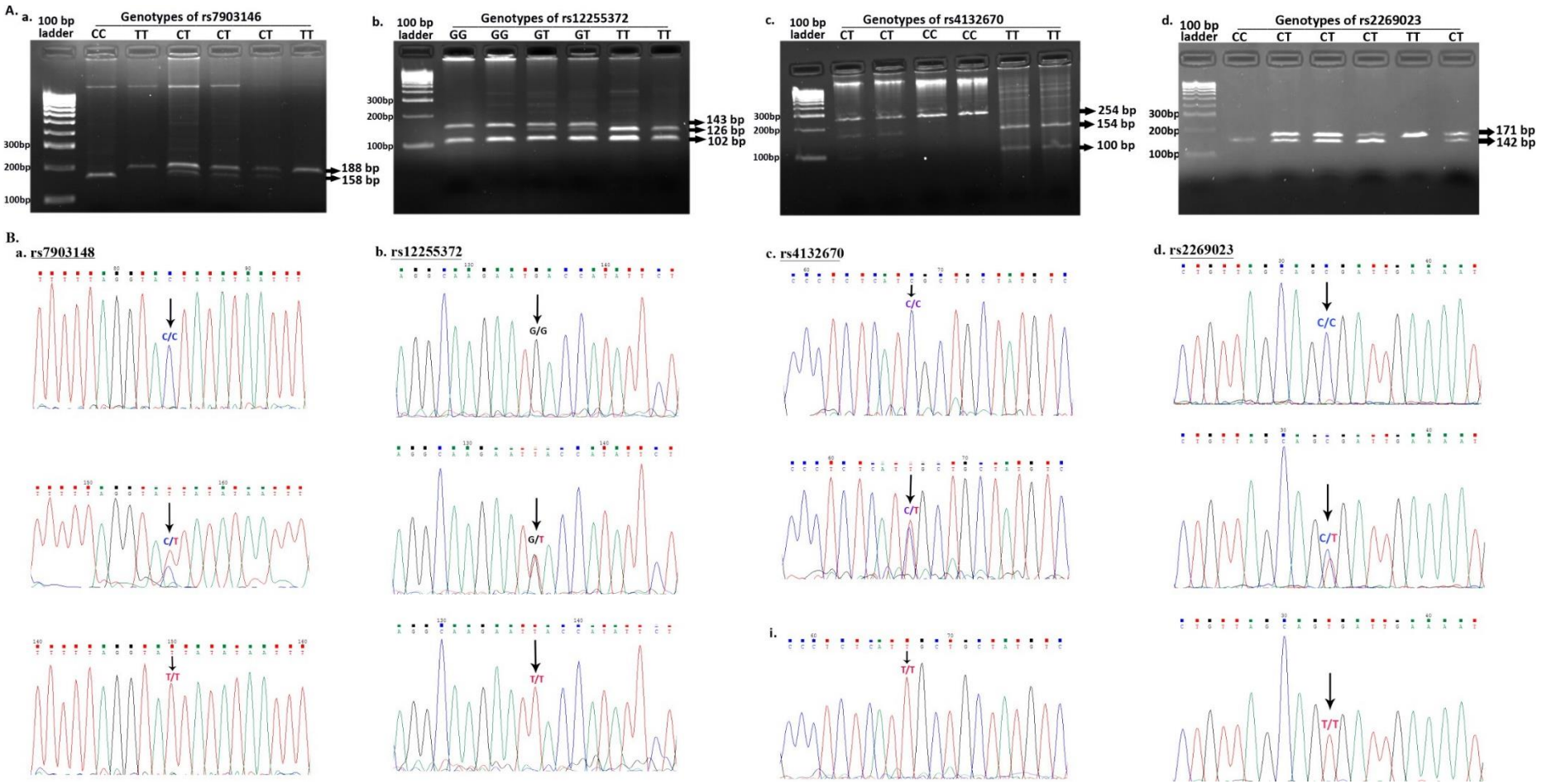

Fig. 5: Genotyping of selected SNPs of *TCF7L2* (rs7903146, rs12255372, rs4132670) and *PCSK2* (rs2269023) by PCR-RFLP method (restriction fragment length polymorphism) and direct DNA sequencing. A. Representative gel photograph showing different genotypes for rs7903146 (CC-158bp, CT- 188bp+158bp, TT-188bp), rs12255372 (GG-126bp+102bp, GT-143bp+126bp+102bp, TT-126bp +102bp), rs4132670 (CC-254bp, CT-154bp+100bp, TT-254bp+154bp+100bp) and rs2269023 (CC-142bp, CT-171bp+142bp, TT-171bp). B. Representative chromatograms of the studied SNPs showing sequencing validation results of PCR-RFLP analysis. Arrow indicated the polymorphic locus.

Table 8A: Allelic frequencies of *TCF7L2* and *PCSK2* variants (SNPs) in the studied groups.

Gene	SNP	Alleles	Allelic frequency		Odds ratio (95% CI)	p value[1]
			Case	Control		
TCF7L2	rs12255372	G	83(73%)	200(87.7%)	Ref=1.0	0.0008*
		T	31(27%)	28(12.3%)	2.67 (1.51-4.72)	
	rs7903146	C	75(66%)	165(72%)	Ref=1.0	0.25
		T	39(34%)	63(28%)	1.36 (0.83-2.2)	
	rs4132670	C	80(70%)	192 (84%)	Ref=1.0	0.002*
		T	34(30%)	36 (16%)	2.26 (1.32-3.87)	
PCSK2	rs2269023	C	58(51%)	141(61.8%)	Ref=1.0	0.063*
		T	56(49%)	87(38.2%)	1.56(0.99-2.46)	

*p value significant; [1]p value calculated by Fischer‟s exact test; SNP, single nucleotide polymorphism.

Table 8B: Genotypic distribution of studied variants (SNPs) and their association with GDM in respective models

Gene	SNP	Genotypes	Genotypic frequency		p value[1]	Models		Odds ratio[‡] (95% CI)	Odds ratio[#] (95% CI)	p value[2]
			Case	Control						
TCF7L2	rs12255372	GG	72 (63%)	176 (77%)	0.005*	Co-dominant	GG vs. GT	1.73(1.03-2.91)	1.75 (1.03-2.95)	0.037*
		GT	34(30%)	48(21%)			GG vs. TT	4.89(1.43-16.75)	4.97(1.41-17.55)	0.013*
		TT	8(7%)	4(2%)		Dominant	GG vs. GT+TT	1.97(1.21-3.22)	1.99 (1.22-3.28)	0.006*
						Recessive	GG+GT vs. TT	4.23(1.25-14.35)	4.47(1.29-15.49)	0.018*
	rs7903146	CC	50(44%)	114(50%)	0.036*	Co-dominant	CC vs. CT	1.09 (0.68-1.76)	1.10(0.67-1.80)	0.68
		CT	49(43%)	102(45%)			CC vs. TT	2.85 (1.24-6.53)	2.93(1.26-6.80)	0.012*
		TT	15(13%)	12(5%)		Dominant	CC vs. CT+TT	1.28(0.82-2.01)	1.30(0.82-2.06)	0.26
						Recessive	CC+CT vs. TT	2.73(1.23-6.04)	2.85(1.26-6.39)	0.011*
	rs4132670	CC	59(52%)	163(71%)	0.0003*	Co-dominant	CC vs. CT	2.05 (1.25-3.36)	2.01(1.22-3.32)	0.006*
		CT	43(38%)	58(26%)			CC vs. TT	4.74 (1.78-12.6)	4.66(1.70-12.77)	0.003*
		TT	12(10%)	7(3%)		Dominant	CC vs. CT+TT	2.34(1.47-3.73)	2.31(1.44-3.71)	0.001*
						Recessive	CC+CT vs. TT	3.71(1.42-9.71)	3.91(1.47-10.44)	0.006*
PCSK2	rs2269023	CC	31(27%)	86(38%)	0.018*	Co-dominant	CC vs. CT	1.36 (0.81-2.3)	1.66(0.95-2.93)	0.07
		CT	54(47%)	110(48%)			CC vs. TT	2.51 (1.31-4.81)	2.69(1.38-5.25)	0.004*
		TT	29(26%)	32(14%)		Dominant	CC vs.CT+TT	1.62(0.99-2.65)	1.79(1.08-2.97)	0.024*
						Recessive	CC+CT vs.TT	2.09(1.19-3.67)	1.86(1.04-3.34)	0.037*

*p value significant.

[1]p value calculated by chi-square test. [2]p value calculated by logistic regression analysis.

[‡]Odds ratio unadjusted. [#] Odds ratio adjusted for age, BMI, acanthosis nigricans and family history of diabetes.

Association of variants with fasting serum pro-insulin and pro-insulin:C-peptide ratio

This was done to assess the impact of *TCF7L2* and *PCSK2* variants on insulin synthesis/ processing pathway. In GDM women, average pro-insulin level (pmol/litre) and pro-insulin: C-peptide ratio (%) was significantly higher than non-GDM control (Figure-6A). When case and control subjects were subdivided in accordance to genotypes of studied variants, individuals carrying GT and TT for rs12255372 showed significantly higher serum pro-insulin level ($p<0.0001$) and pro- insulin: C-peptide ratio ($p<0.0001$) than those with GG (Figure-6B); similar trend was also recorded in individuals having CT and TT genotypes than those with CC for rs4132670 ($p=0.004$ and 0.003 respectively) and for rs7903146 ($p=0.003$ and 0.006 respectively). However in rs2269023, although individuals with CT and TT genotypes showed higher values for proinsulin level and proinsulin: C-peptide ratio than carriers of CC, but the difference did not reach to statistical significance ($p=0.158$ and 0.095).

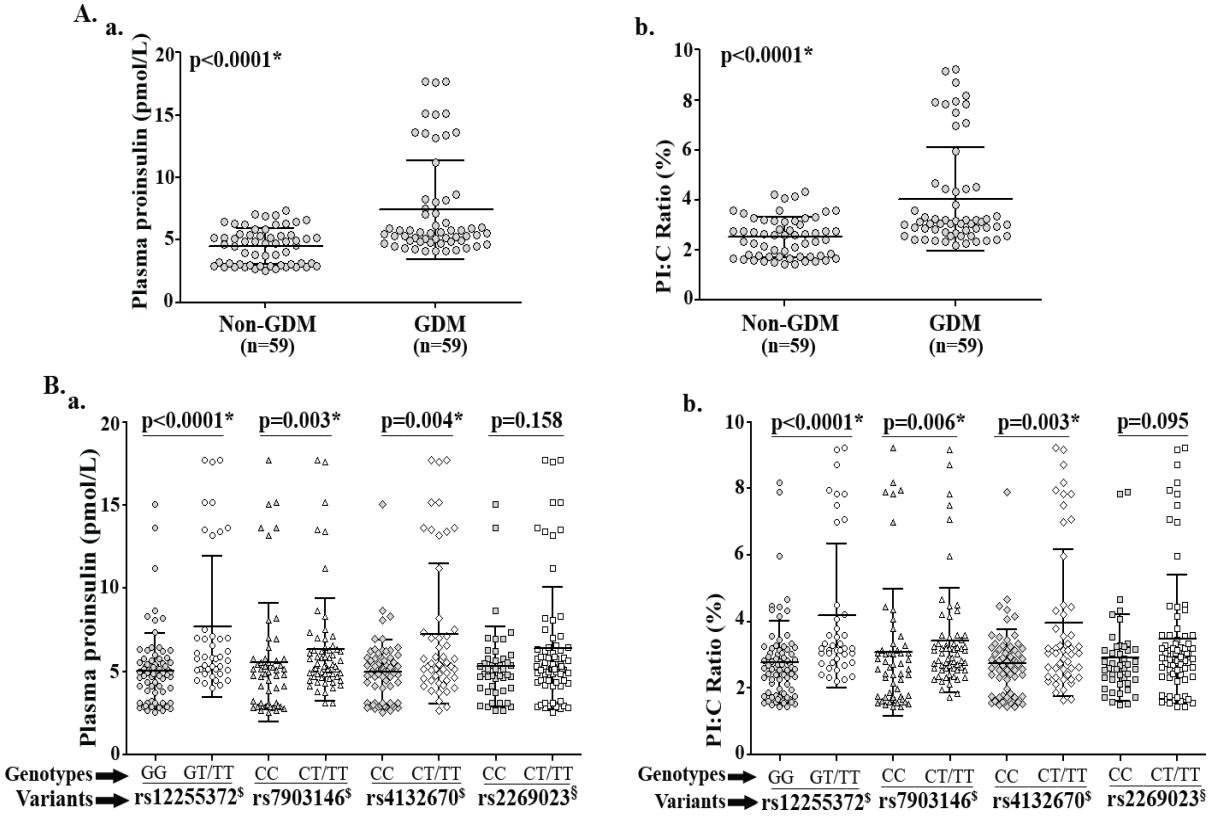

Fig 6: A. Comparison of fasting serum pro-insulin level (a) and pro-insulin: C-peptide ratio (b) between GDM and non-GDM subjects. B. Association of *TCF7L2* and *PCSK2* gene variants with fasting serum pro-insulin level (a) and pro-insulin: C-peptide ratio (b) in study participants. PI, pro-insulin; C, C-peptide; $, variants of *TCF7L2*; §, variant of *PCSK2*; *p value significant

ii. Genetic variants associated with insulin secretion pathway

Variant of KCNJ11 and ABCC8: For each of the variant rs5219 of *KCNJ11* and rs757110 of *ABCC8*, three possible genotypes were readily found in our study subjects (Figure-7). In case and control groups genotypic distributions of the SNPs did not deviate significantly (p>0.05) from Hardy-Weinberg equilibrium.

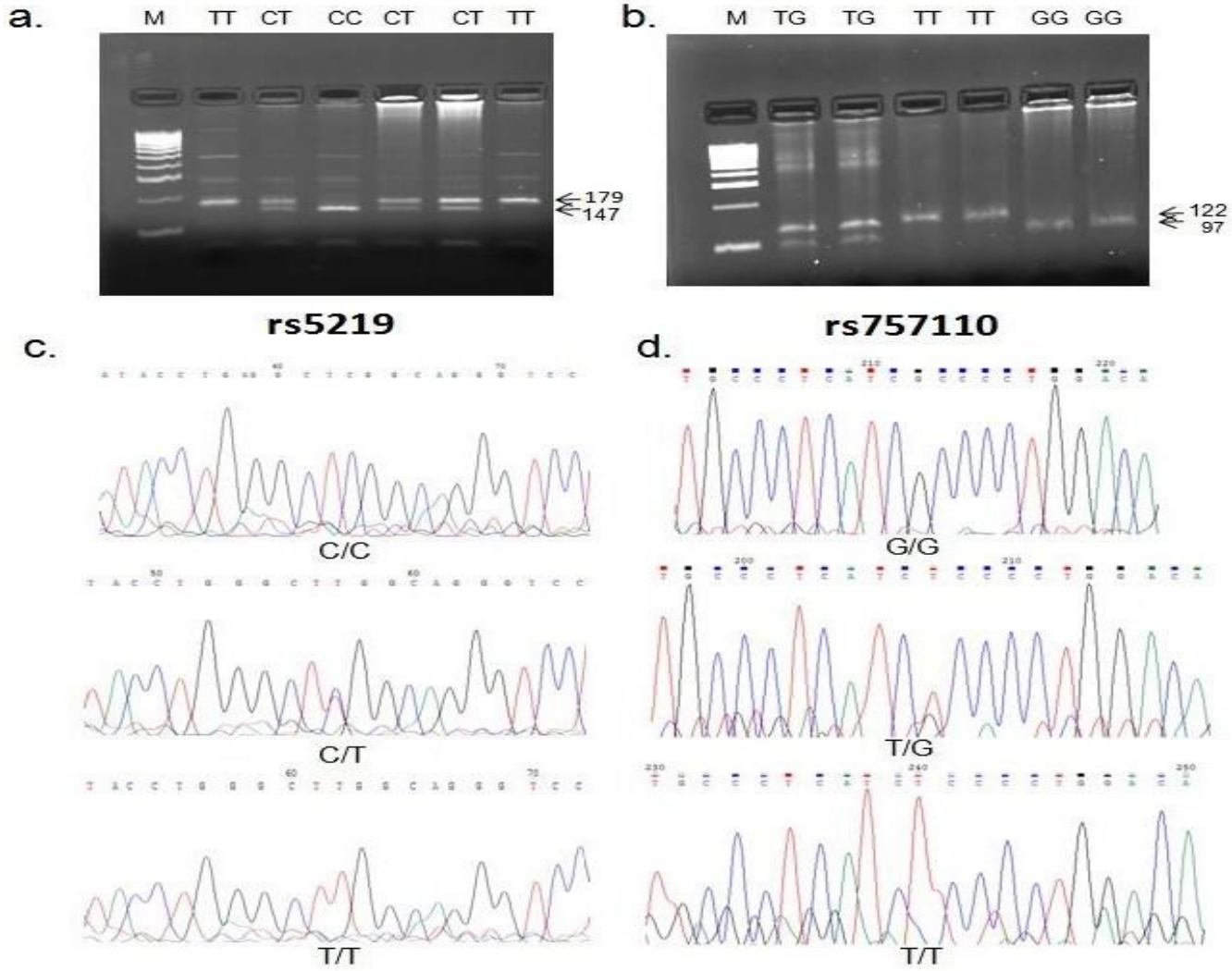

Fig7: Representative gel electrophoresis photographs of rs5219 & rs757110 polymorphism of *KCNJ11 & ABCC8* (a&b). Analysis of single nucleotide polymorphism (SNP) using Sanger sequencing of rs5219 & rs757110 (d&e). M,100bp molecular marker.

Comparison of allelic distribution among case and control: In rs5219 (C/T), frequency of minor allele (T) was considerably higher in GDM individual than control, but the difference was not found to be statistically significant (37%, 42/114 vs. 28%, 63/228; p=0.11) (Table- 9a). In rs757110 (C/G), frequency of minor allele (G) was marginally significantly higher in GDM case than control (33%, 38/114 vs. 23%, 52/228; p=0.05); therefore, G allele of rs757110 seemed to be the risk allele for GDM (OR= 1.69; 95% CI=1.03-2.78) in our studied population.

Comparison of genotypic distribution among case and control: In rs5219, significantly higher frequency of case individuals were presented with TT genotype in comparison to control (18%, 21/114 vs. 7%, 16/228; p=0.006); individuals carrying TT genotype showed about 3 fold higher risk for GDM in co-dominant (CC vs. TT; OR=3.2, 95%CI=1.51-6.79, p=0.002) and recessive (CC+ CT vs. TT; OR=3.23, 95%CI=1.59-6.56, p=0.001) genetic models after adjusting for potential confounders like age, BMI, acanthosis nigricans and diabetic family history (Table-9b). For rs757110, in line with the result showing „G" as the risk allele in studied population, GG genotype was presented in significantly higher frequency in case compared to control (10%, 11/114 vs. 3%, 7/228; p=0.007); GG genotype also showed significant association with increased GDM risk in co-dominant (TT vs. GG; OR=4.16, 95%CI=1.48-11.69, p=0.007) and recessive models (TT+GT vs. GG; OR=3.3, 95%CI=1.23-8.85, p=0.01) and the combined genotype TG+GG in dominant genetic model (CC vs. TG+GG; OR=1.67, 95%CI =1.05-2.65, p=0.03) after adjustment for potential confounders

Variants of *MTNR1B:*

For *MTNR1B* two noncoding variants namely rs1387153 and rs10830963 were selected and three possible genotypes of each variant were successfully detected in our studied samples (Figure-8). In our study subjects, no significant deviation (p>0.05) of genotypic distribution of the studied variants was found from Hardy-Weinberg equilibrium.

Allelic and genotypic distribution in case vs. control: Among the two SNPs of *MTNR1B*, allelic and genotypic distribution of rs10830963 showed significant difference between GDM and non-GDM individuals. For rs10830963 „G" seemed to be the risk allele in our studied population, as presented with significantly higher frequency in case compared to control subjects (40%, 46/114 vs. 27%, 61/228; p=0.011) (Table-10a). Similarly, association of homozygous risk genotype (GG) was found with more than 3-fold higher disease risk than dominant homozygous genotype (CC vs. GG, OR=3.33, p=0.001) and about 3-fold higher risk than the combined genotype CC+CG (CC+CG vs. GG, OR=2.66, p=0.005) (Table-10b).

Table-9a: Allelic frequencies of *KCNJ11* and *ABCC8* variants (SNPs) in the studied groups

Gene	SNP	Alleles	Allelic frequency		Odds ratio (95% CI)	p value[1]
			Case	Control		
KCNJ11	rs5219	C	72(63%)	165(72%)	Ref=1.0	0.11
		T	42(37%)	63(28%)	1.53 (0.95-2.47)	
ABCC8	rs757110	T	76(67%)	176(77%)	Ref=1.0	0.05
		G	38(33%)	52(23%)	1.69(1.03-2.78)	

Table-9b: Genotypic distribution of studied variants (SNPs) and their association with GDM in respective models

Gene	SNP	Genotypes	Genotypic frequency		p value[1]	Model		Odds ratio[‡] (95% CI)	Odds ratio[#] (95% CI)	p value[2]
			Case	Control						
KCNJ11	rs5219	CC	52 (46%)	118 (52%)	0.006*	Co-dominant	CC vs. CT	0.99(0.61-1.62)	0.95 (0.58-1.57)	0.844
		CT	41(36%)	94(41%)			CC vs. TT	2.98(1.44-6.17)	3.20(1.51-6.79)	0.002*
		TT	21(18%)	16(7%)		Dominant	CC vs. CT+TT	1.28(0.82-2.01)	1.24 (0.78-1.96)	0.36
						Recessive	CC+CT vs. TT	2.99(1.49-5.99)	3.23(1.59-6.56)	0.001*
ABCC8	rs757110	TT	50(44%)	131(58%)	0.007*	Co-dominant	TT vs. TG	1.54(0.96-2.47)	1.50(0.93-2.43)	0.094
		TG	53(46%)	90(39%)			TT vs. GG	4.1(1.51-11.21)	4.1(1.48-11.69)	0.007*
		GG	11(10%)	7(3%)		Dominant	TT vs. TG+GG	1.73(1.09-2.72)	1.67(1.05-2.65)	0.030*
						Recessive	TT+TG vs. GG	3.37(1.27-8.95)	3.30(1.23-8.85)	0.018*

*p value significant.

[1]p value calculated by chi-square test. [2]p value calculated from logistic regression analysis.

[‡]Odds ratio unadjusted. [#] Odds ratio adjusted for age, BMI, acanthosis nigricans and family history of diabetes.

Moreover, individuals having at least one risk allele (GG+CG) were also at higher disease risk than carriers of dominant homozygous genotype (CC vs. GG+CG, OR=1.84, p=0.01). However, for rs1387153, no significant difference in allelic and genotypic frequencies was found between case and control individuals (Table-14a and 14b). Similarly, no significant association of the SNP genotypes was established with disease risk in the studied genetic models.

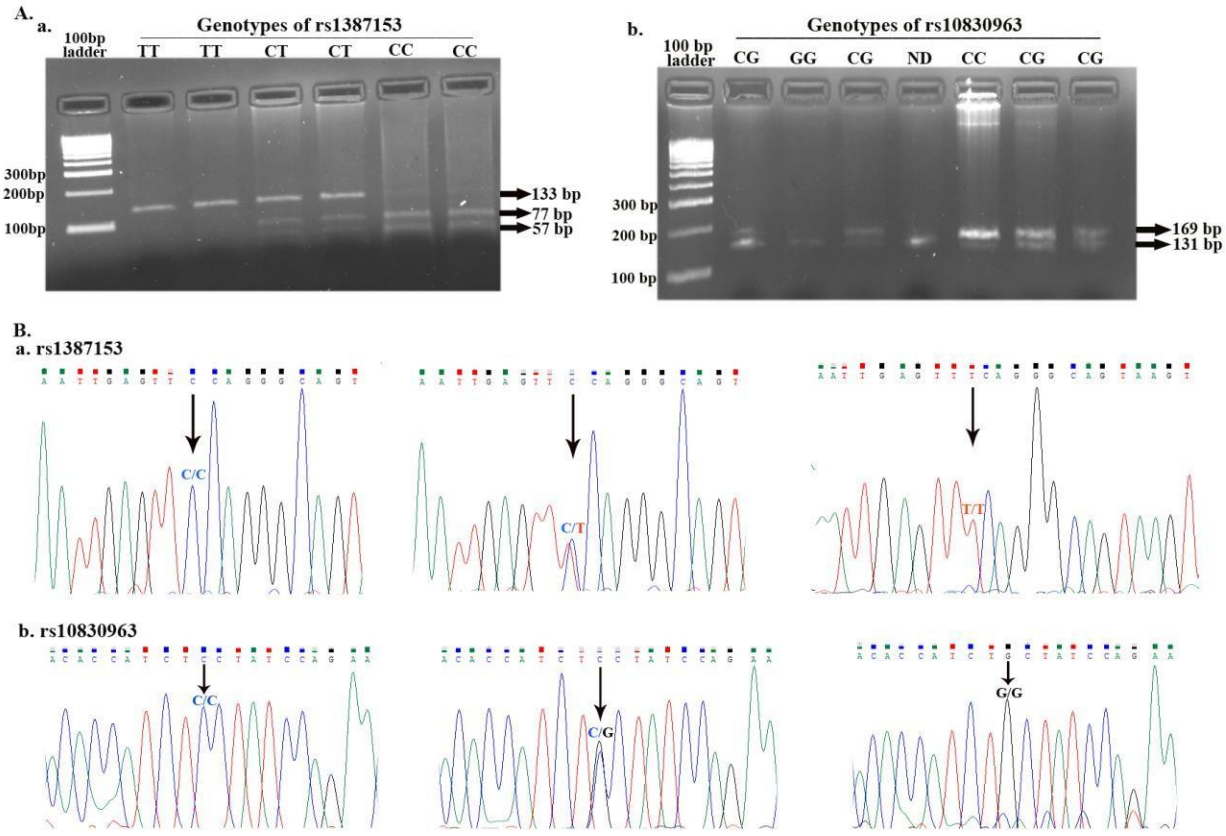

Figure-8: Genotyping of selected SNPs of *MTNR1B* (rs1387153 & rs10830963) by PCR-RFLP method (restriction fragment length polymorphism) and direct DNA sequencing. A. Representative gel photograph showing major restriction fragments for different genotypes for rs1387153 (TT-133bp CC-77bp+55bp, CT-133bp+77bp+55bp), and rs10830963 (CC-169bp, GG-133bp, GT-169bp+133bp). B. Representative chromatograms of the studied SNPs showing sequencing validation results of PCR-RFLP analysis. Arrow indicated the polymorphic locus.

Table-10a: Allelic frequencies of *MTNR1B* variants (SNPs) in the studied groups

Gene	SNP	Alleles	Allelic frequency		Odds ratio (95% CI)	p value[1]
			Case	Control		
MTNR1B	rs1387153	C	65(57%)	141(62%)	Ref=1.0	0.41
		T	49(43%)	87(38%)	1.22 (0.77-1.93)	
MTNR1B	rs10830963	C	68(60%)	167(73%)	Ref=1.0	0.011*
		G	46(40%)	61(27%)	1.85(1.15-2.98)	

*p value significant
[1]p value calculated by Fischer"s exact test. SNP, single nucleotide polymorphism.

Table-10b: Genotypic distribution of studied variants (SNPs) and their association with GDM in respective models

Gene	SNP	Genotypes	Genotypic frequency		p value[1]	Model		Odds ratio[‡] (95% CI)	Odds ratio[#] (95% CI)	p value[2]
			Case	Control						
MTNR1B	rs1387153	CC	44(39%)	88(39%)	0.063	Co-dominant	CC vs. CT	0.79(0.48-1.32)	0.82(0.49-1.37)	0.439
		CT	42(37%)	106(46%)			CC vs. TT	1.65(0.89-3.05)	1.58(0.85-2.95)	0.149
		TT	28(24%)	34(15%)		Dominant	CC vs. CT+TT	1.00(0.63-1.59)	0.99(0.624-1.59)	0.984
						Recessive	CC+CT vs. TT	1.86(1.06-3.26)	1.75(0.99-3.09)	0.053
MTNR1B	rs10830963	CC	44(39%)	124(54%)	0.002*	Co-dominant	CC vs. CG	1.57(0.96-2.58)	1.56(0.94-2.57)	0.08
		CG	48(42%)	86(38%)			CC vs. GG	3.44(1.69-7.02)	3.33(1.61-6.86)	0.001*
		GG	22(19%)	18(8%)		Dominant	CC vs.CG+GG	1.89(1.19-3.00)	1.84(1.16-2.93)	0.010*
						Recessive	CC+CG vs. GG	2.79(1.43-5.45)	2.66(1.35-5.23)	0.005*

*p value significant.
[1]p value calculated by chi-square test. [2]p value calculated from logistic regression analysis.
[‡]Odds ratio unadjusted. [#] Odds ratio adjusted for age, BMI, acanthosis nigricans and family history of diabetes

Association of *KCNJ11* and *ABCC8* and *MTNR1B* variants with insulin resistance and β-cell function

Fasting plasma insulin level was measured in 59 GDM and equal number of control subjects and associated biochemical parameters were analyzed (Figure-9). GDM subjects were presented with significantly higher median value for fasting insulin (μU/ml) in comparison to control (15.25 vs. 13.79, p<0.0001). Parameter of insulin resistance was found to be significantly higher (HMA-IR, 3.63 vs. 2.68, p<0.0001) and parameters of insulin sensitivity (QUICKI, 0.3157 vs. 0.3293, p<0.0001) and β-cell function (HOMA-B, 53.40 vs. 61.94, p<0.0001) was significantly lower in GDM subjects compared to the control.

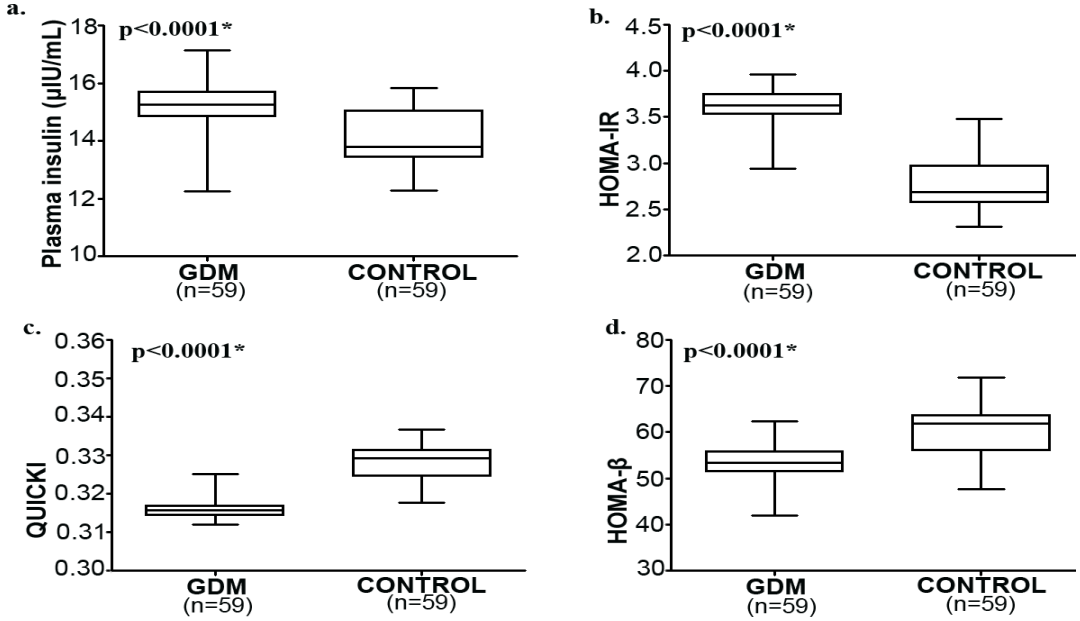

Figure-9: Comparison of fasting plasma insulin level (a), index of homeostasis model assessment of insulin resistance (HOMA-IR) (b), quantitative insulin sensitivity check index (QUICKI) (c) and index of homeostasis model assessment of β-cell function (HOMA-β) (d) in GDM and control subjects. GDM, gestational diabetes mellitus; n=number of samples analyzed.

When case as well as control subjects were grouped under genotypes of rs5219, rs757110, rs1387153 and rs10830963, no significant association of the SNPs was found with fasting insulin level and parameters of insulin resistance /sensitivity (HOMAIR/QUICKI) (Table-11a and b). But among the SNPs, association of rs5219, rs757110 was found significant with the parameter of β-cell function (HOMA-β) i.e. individuals of recessive homozygous genotypes for rs5219/rs757110 showed reduced HOMA- β values that showed a continuously and significantly increasing trend across the carriers of heterozygous and dominant homozygous genotypes (rs5219, TT/51.52 vs. CT/55.03 vs. CC/58.98, p=0.001; rs757110, GG/49.58 vs. TG/54.72 vs. TT/59.19, p<0.0001). However, HOMA-β values remained comparable among carriers of different genotypes for rs1387153 and rs10830963 of *MTNR1B* in our studied population.

Table 11a: Association of rs5219 and rs757110 with quantitative traits (Glucose and Insulin metabolism) in studied subjects (case and control)

Gene	SNP	Genotypes	FI (µIU/mL)		HOMA-IR		HOMA-β		QUICKI	
			Median (range)	p ‡	Median (range)	p ‡	Median (range)	p ‡	Median (range)	p ‡
KCNJ11	rs5219 (C/T)	CC (n=55)	14.81(12.28-17.14)	0.913	2.87(2.31-3.96)	0.065	58.98(47.68-	0.001*	0.326(0.312- 0.336)	0.064
		CT (n=49)	14.99(12.52-16.51)		3.18(2.52-3.84)		55.03(48.95-66.87)		0.321(0.313-0.332)	
		TT (n=14)	14.93(12.25-16.24)		3.47(2.94-3.85)		51.52(41.88-		0.317(0.313-0.325)	
ABCC8	rs757110 (T/G)	TT (n=56)	14.82(12.28-17.14)	0.784	3.12(2.45-3.96)	0.239	59.19(47.68-	<0.0001*	0.322(0.312-0.333)	0.239
		TG (n=54)	14.98(12.25-16.51)		3.29(2.31-3.84)		54.72(41.88-70.78)		0.320(0.313-0.336)	
		GG (n=8)	14.60(12.79-15.47)		3.46(3.07-3.85)		49.58(43.87-		0.318(0.313-0.323)	

Table 11b: Association of rs1387153 and rs10830963 with quantitative traits (Glucose and Insulin metabolism) in studied subjects (case and control)

Gene	SNPs	Genotypes	FI (µIU/mL)		HOMA-IR		HOMA-β		QUICKI	
			Median (range)	p ‡	Median (range)	p ‡	Median (range)	p ‡	Median (range)	p ‡
MTNR1B	**rs1387153 (C/T)**	CC (n=46)	14.54(12.25-17.14)	0.159	3.07(2.45-3.96)	0.083	55.56(41.88-70.78)	0.4084	0.323(0.312-0.333)	0.083
		CT (n=49)	14.84(12.52-16.88)		3.17(2.31-3.90)		57.74(48.88-71.92)		0.321(0.312-0.336)	
		TT (n=23)	15.25(13.61-16.99)		3.56(2.51-3.93)		55.03(45.98-64.79)		0.316(0.312-0.332)	
	rs10830963 (C/G)	CC (n=55)	14.34(12.25-16.24)	0.083	3.06(2.31-3.84)	0.088	56.40(41.88-71.92)	0.9331	0.323(0.313-0.336)	0.091
		CG (n=41)	14.91(12.57-17.14)		3.38(2.45-3.96)		55.90(45.98-71.58)		0.318(0.312-0.333)	
		GG (n=22)	15.26(12.52-16.99)		3.54(2.45-3.93)		55.47(49.51-68.94)		0.3168(0.3124-0.3337)	

*p value significant. p ‡ value calculated by Kruskal-Wallis test. FI, fasting plasma insulin. HOMA-IR, homeostasis model assessment of insulin resistance QUICKI, quantitative insulin sensitivity check index. HOMA- β, homeostasis model assessment of β-cell function.

Objective 2: Analysis of expression profile of selected genes in placental tissue to explore the molecular basis of disease outcome

Sociodemographic and clinical features of case (n=70) and control (n-81) individuals were summarized in Table-12. Control (non-GDM) individuals were matched with cases (GDM) for maternal age, gestational age and BMI.

Table-12: Descriptive characteristics of mother and neonates

		GDM (n=70)	CONTROL (n=81)	p value
Maternal characteristics[1]				
Maternal Age (year)		24.33 ± 4.76	24.16 ± 3.85	0.88[$]
Pre gestational BMI (Kg/m2)		24.22 ± 3.06	23.44 ± 3.19	0.38[$]
2-hour OGTT (mg/dL)		164.8 ± 39.62	109.8 ± 9.72	<0.0001[*$]
Gestational Age at the end of pregnancy (weeks)		36.6± 2	36.95 ± 1.9	0.314[$]
Neonatal Complications[2]				
Macrosomia	Yes	12 (17%)	1 (1%)	0.0006[*#]
	No	58 (83%)	80 (99%)	
Neonatal hypoglycaemia	Yes	13 (19%)	2 (2%)	0.001[*#]
	No	57 (81%)	79 (98%)	
Hyperbilirubinaemia	Yes	11 (15.7%)	11 (13.6%)	0.82[#]
	No	59 (84.2%)	70 (86.4%)	
Hypothermia	Yes	3 (4%)	5 (6%)	0.72[#]
	No	67 (96%)	76 (94%)	
Respiratory distress	Yes	8 (11%)	4 (5%)	0.226
	No	62 (89%)	77 (95%)	
Hypocalcaemia	Yes	2 (3%)	1 (1%)	0.59
	No	68 (97%)	80 (99%)	
NICU admission	Yes	17 (24%)	8 (10%)	0.026[**]
	No	53 (76%)	73 (90%)	

BMI, body mass index; OGTT, oral glucose tolerance test; NICU, newborn intensive care unit; [1]continuous variables presented as mean ± standard deviation; [2]categorical variable presented as number (percentage); *p value significant; [#]p calculated by Fisher‟s exact test; [$]p calculated by Mann-Whitney test Among complications documented in neonates, neonatal hypoglycaemia (19%, 13/70) and macrosomia (17%, 12/70) was found to be most common as GDM associated outcome compared to that of non-GDM control (2%, 2/70 and 1%, 1/70 respectively) and the differences were statistically significant (p=0.001 and p=0.0006 respectively). Among other neonatal abnormalities, incidence of jaundice, respiratory distress, hypothermia and hypocalcaemia were also documented in our study, but in relatively lower frequencies that remained comparable among the newborn of GDM and non-GDM mothers. However, in GDM associated pregnancy, incidence of NICU admission was found to be significantly higher than that of non-GDM control (24%, 17/70 vs. 10%, 8/81; p=0.026).

Placental histopathogylogical abnormalities

Approximately nine different types of histopathological abnormalities of human placenta, were successfully detected in our studied samples and hence included in the analysis (Figure-10, Table-13).

Table 13: Comparison of placental histopathology among GDM and control subjects

Histological abnormalities	GDM (n=70)	CONTROL (n=81)	*p*-value[#]
Villous immaturity	27 (38.5%)	30 (37.04%)	0.86
Chorangiosis	25 (35.7%)	14 (17.3%)	0.014[*]
Villous edema	23 (32.86%)	11 (13.58%)	0.0061[*]
Fibrinoid deposition	26 (37.14%)	23 (28.39%)	0.2969
Vascular Congestion	5 (7.14%)	4 (4.9%)	0.73
Increased no. of syncytial knots	9 (12.9%)	2 (2.5%)	0.024[*]
Thickening of BM	10 (14.3%)	3 (3.7%)	0.038[*]
Calcification	3 (4.29%)	2 (2.46%)	0.66
NRBC	9 (12.86%)	3 (3.7%)	0.067

Values presented in number (%). NRBC, *Nucleated red blood cells*; BM, Basement membrane * p value significant; #p value calculated by Chi-square test.

Among histological abnormalities, chorangiosis, villous immaturity and fibrinoid deposition were found most frequently, while vascular congestion and calcification appeared rarely in our studied population irrespective of GDM case and non-GDM categories. While doing comparison between case and control, it was found that chorangiosis (case-35.7% vs. control-17.3%, p=0.014), villous edema (case-32.86% vs. control-13.58%, p=0.006), syncytial knot (case-12.9% vs. control-2.5%, p=0.024) and thickening of villous basement membrane (case-14.3% vs. control-3.7%, p=0.038) were detected in significantly higher frequency in placenta of GDM women than that of non-GDM control.

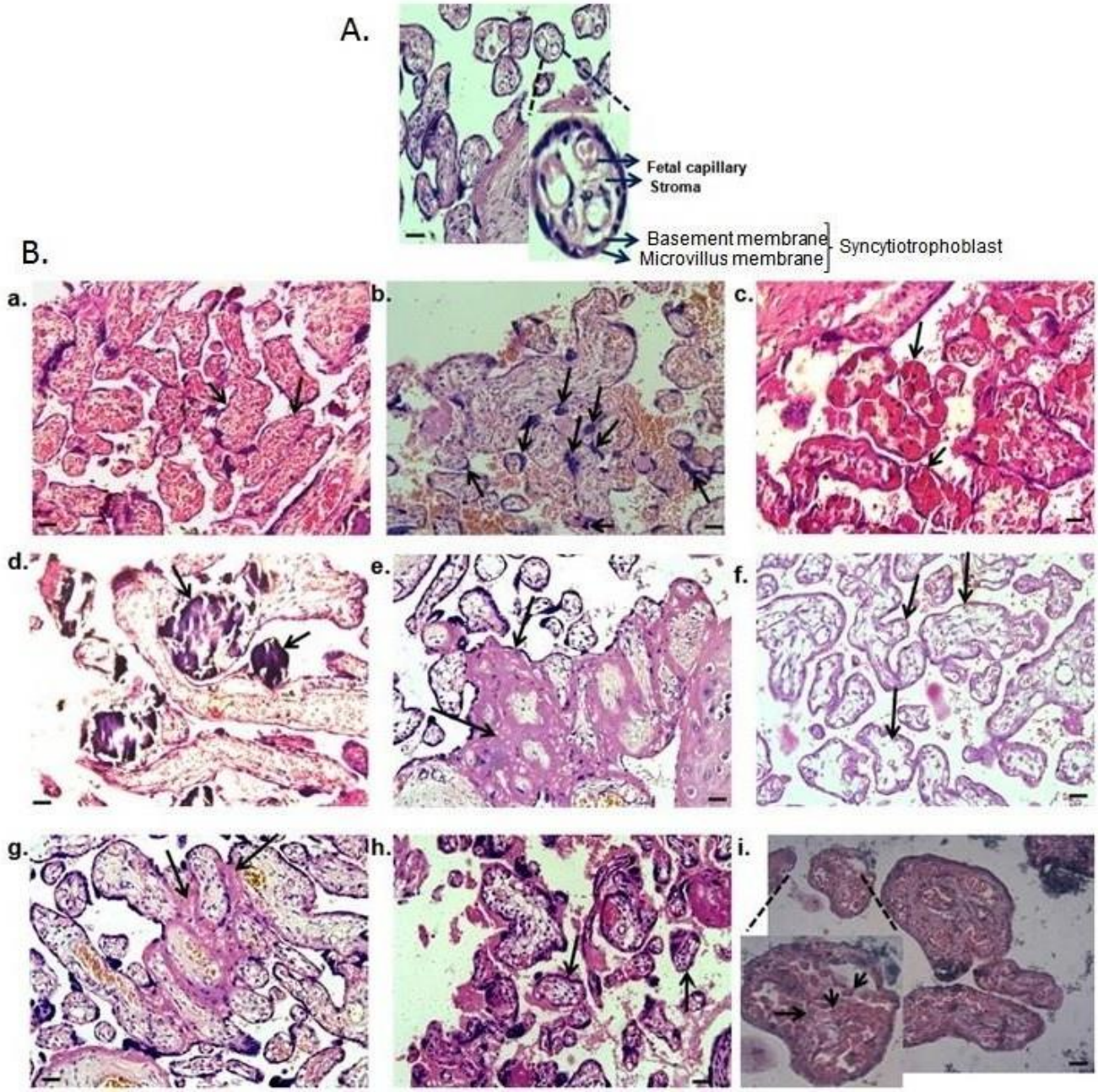

Fig 10: Representative photographs of different histological abnormalities in placenta. (A) Normal chorionic villi, (B) Different abnormalities of term placenta- (a) Chorangiosis (Excess growth of fetal blood vessels into chorionic villi and can be diagnosed as presence of at least 10 villi each with minimum 10 blood vessels in one low power microscopic field); (b) Syncytial Knotes (Presence of aggregated syncytial nuclei on villous syncytiotrophoblast layer); (c) Vascular Congestion (Congestion or obstruction of blood vessels in chrionic villi as evident from loss of plasma between erythrocytes, extravasations, signs of hemolysis etc.); (d) Calcification (Deposition of calcium phosphate minerals mainly in intravillous space including basement membrane of chorionic villi); (e) Fibrosis (Increased deposition of fibrous tissue in villous stroma and concordantly decreased fetal capillarization with respect to gestational age) ; (f) Villous edema (Presence of open space in cytoplasm of intervillous cells and within villous interstitium due to accumulation of fluid) ; (g) Fibrinoid deposition (Presence of cellular, eosinophilic and homogeneous objects in intra and extravillous space); (h) Immature Villi (increased proportion of immature villi in comparison to terminal villi in placenta with respect to gestational age); (i) NRBC, Nucleated red blood cells (Presence of nucleated fetal RBC in villous blood vessels). Original magnification X20, scale bar 20µm

Expression of glucose transporters (GLUTs)

Expression analysis was done both at mRNA as well as protein levels for the selected GLUTs in placental tissue samples of study subjects belonging to GDM and non-GDM categories.

mRNA expression analysis: Among the glucose transporters selected for expression analysis of present study, mRNA of GLUT1, GLUT3, GLUT4 and GLUT8 were readily detected in the term placental samples; this was evident from the amplicon of specific size for each of the mentioned GLUTs in representative gel photograph (Figure-11). However, we failed to find any expression for GLUT-9 and GLUT-12 mRNAs in our placental tissue samples. In quantitative real time PCR, significant fold up-regulation was recorded for GLUT1 ($p<0.0001$) and GLUT3 mRNA ($p<0.0001$) in the placental tissue samples of GDM women compared to that of non-GDM control (Figure-12). However, mRNA expression level of GLUT4 and GLUT8 remained comparable among the placental samples of GDM and non-GDM categories

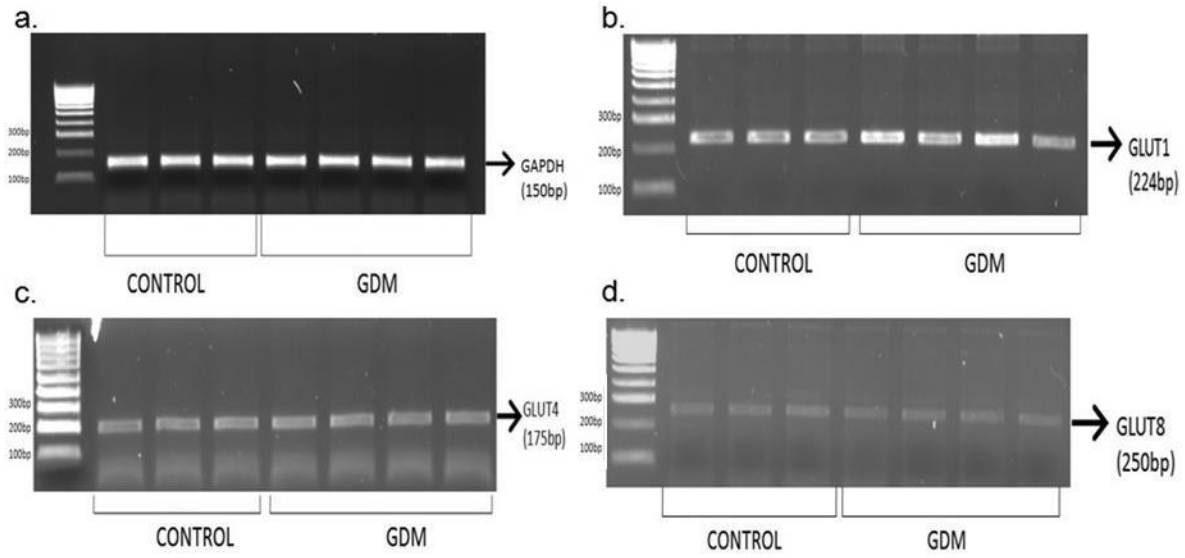

Fig 11: Representative agarose gel photograph of amplicons (real time product) of GAPDH and GLUT1, GLUT4 & GLUT8 (a, b, c & d) in CONTROL and GDM subjects. GDM, gestational diabetes mellitus.

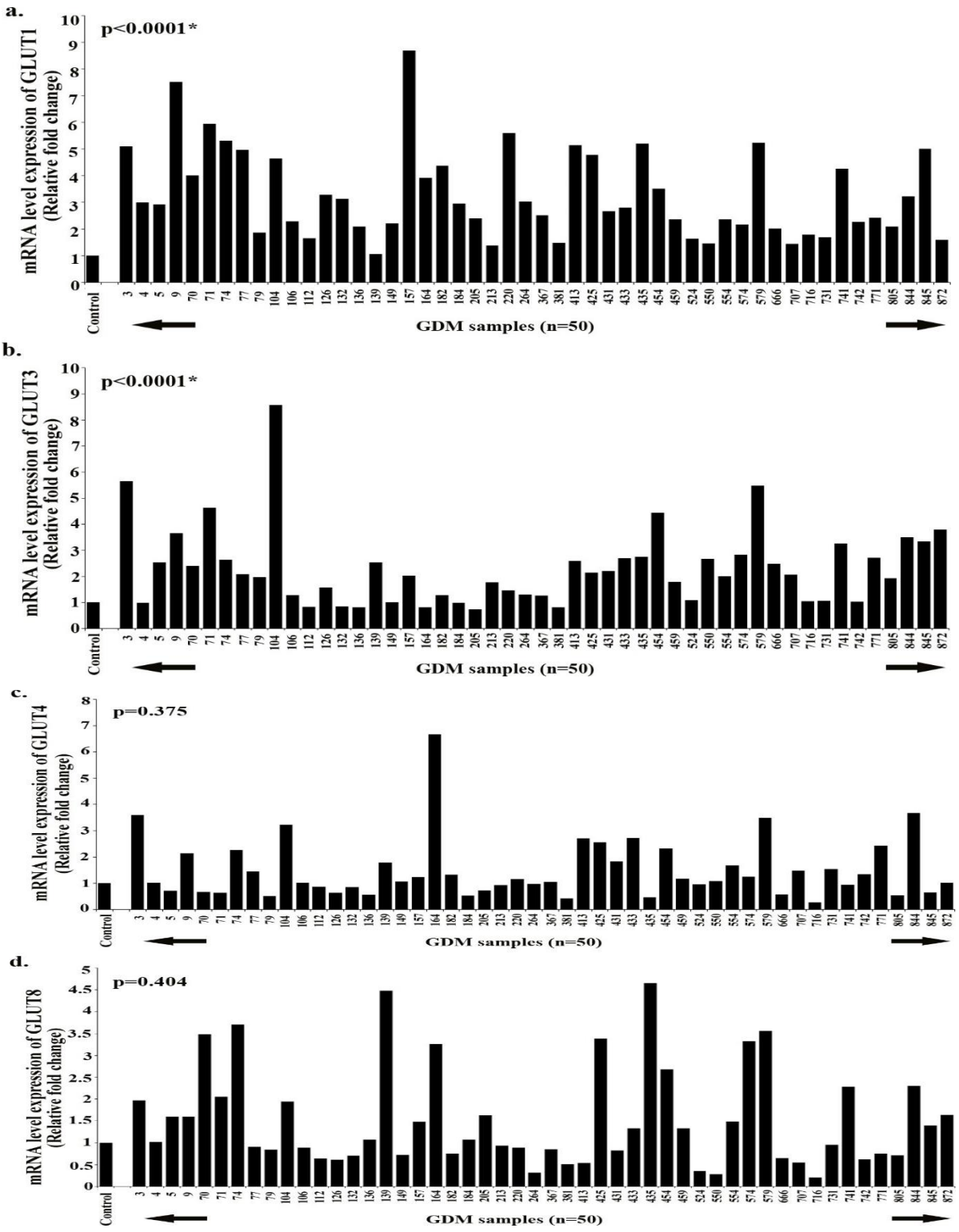

Figure 12: mRNA expression analysis of glucose transporters (GLUTs) by quantitative real time PCR in placental tissue samples of GDM individuals compared to non-GDM control. Bars represent fold change of expression of candidate GLUTs in GDM placenta compared to non-GDM one. *p value significant

Protein expression analysis: GLUT1 & GLUT3 were selected for immunohistochemical analysis as the candidate GLUTs showed significant differential expression at mRNA level in GDM vs. non-GDM placenta

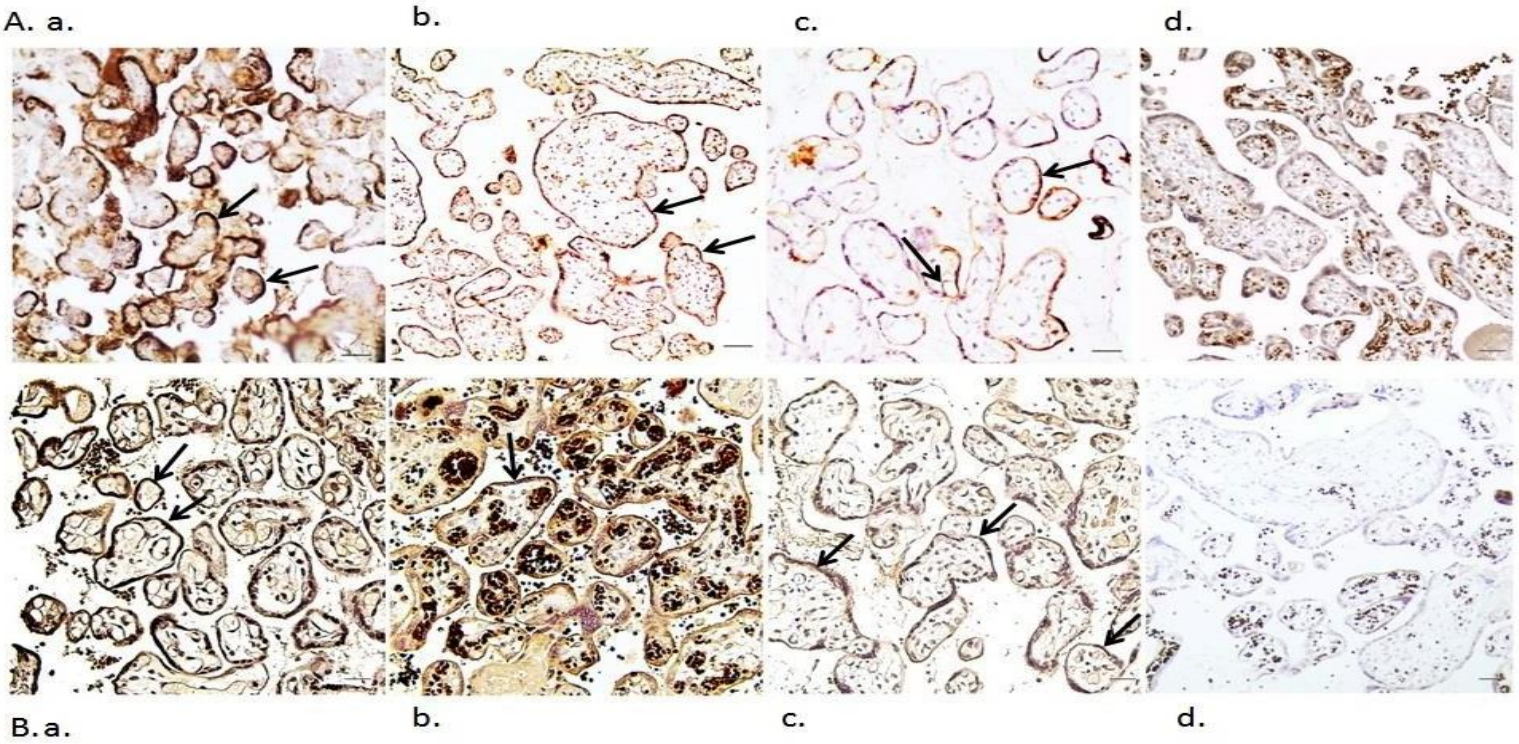

Fig 13: Immunohistochemical analysis of GLUT1 (A) and GLUT3 (B) in human term placenta. A (a- d) indicates the expression of GLUT1 & B (a-d) indicates the expression of GLUT3. Original magnification X20. Scale bar, 40µm

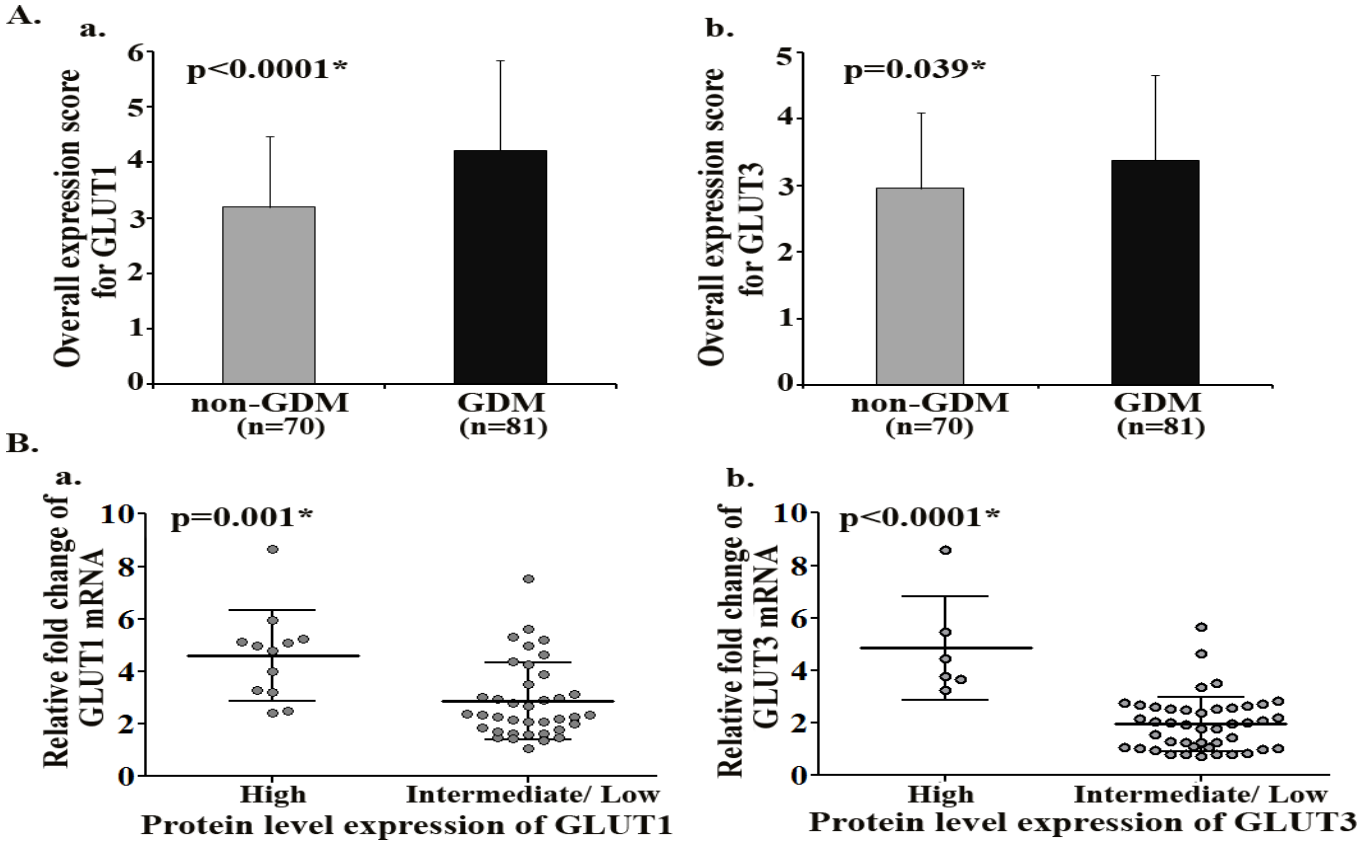

Fig. 14: Comparison of overall expression level of GLUT1 (Aa) and GLUT3 (Ab) proteins among non-GDM versus GDM placental samples. Association of mRNA level expression of GLUT1 and GLUT3 with respective protein levels (Ba and Bb respectively) in GDM samples. n, number of samples. * p value significant

In our study, GLUT1 and GLUT3 proteins were detected in syncytiotrophoblast layer and fetal capillaries of the placental chrionic villi (Figure-13A&B). Furthermore, GLUT1 and GLUT3 were found on both microvillous and basement membranes of a villous structure and expression levels between the membranes were found to be comparable. Overall expression scores for GLUT1 and GLUT3 proteins remained significantly higher in placental tissue of GDM individuals than that of non-GDM control (Figure-14A); moreover, fold up-regulation of GLUT1 and GLUT3 mRNA in GDM placenta showed concordance with the respective protein level expressions (Figure-14B).

Objective 3: Clinicopathological association of disease specific molecular markers

In the present study, association of some genetic markers was found with disease phenotype and therefore their probable contribution in disease pathogenesis was predicted. On the other hand, placental histological changes and expression of selected nutrient transporters in placental tissue were analyzed to understand the pathogenesis of adverse neonatal outcome. Therefore, clinicopathological analysis of the present study was considered under two broad heads.

Association of selected genetic variants with clinicopathological parameters:

In table-14a and 14b, clinicopathological data of GDM and non-GDM subjects categorized according to the genotypes of selected genetic variants of *TCF7L2* and *PCSK2* from insulin synthesis/ processing pathway and *KCNJ11, ABCC8* and *MTNR1B* from insulin secretion pathway was summarized. The values of FBG and PPBG differ significantly among study subjects categorized according to genotypes of rs12255372, rs7903146, rs4132670 of TCF7L2 and rs2269023 of PCSK2; the similar trend was also found for rs5219 of *KCNJ11,* rs757110 for *ABCC8* and rs10830963 of *MTNR1B*. Association of BMI did not remain significant with any of the studied genetic variants of insulin synthesis/processing and secretion pathways; similarly incidence of acanthosis nigricans did not differ significantly among the studied subjects categorized according to genotypes of the variants. However significant association of family history of diabetes was found with rs rs4132670 of *TCF7L2* and rs2269023 of *PCSK2*; but for the other genetic variants included in the study, the association did not reach to statistical significance.

Table 14a: Association of selected genetic variants of selected genes from insulin synthesis pathway with clinicopathological pathway

GENE	SNPs	Genotype	BMI		FPG (mg/dl)		PPBG (mg/dl)		Acanthosis Nigricans			FHD		
			Mean ± SD	p-value	Mean ± SD	p-value	Mean ± SD	p-value	Yes	No	p-value	Yes	No	p-value
TCF7L2	**rs12255372**	GG (248)	24.08±3.19		86.02±8.09		122.9±35.66		134	114		96	152	
		GT (82)	24.22±3.22		89.38±7.41		130±31.24		46	36	0.9171	32	50	0.9788
		TT (12)	24.24±1.62	0.9711	91.83±5.94	0.0004*	158.3±69.19	0.0117*	7	5		5	7	
	rs7903146	CC (164)	24.20±3.27		86.73±7.76		125.9±37.11		96	68		60	104	0.4740
		CT (151)	24.01±2.89		86.65±8.39		122.8±35.47		74	77	0.1578	64	87	
		TT (27)	24.27±3.85	0.9210	91.04±6.54	0.0254*	142.1±39.44	0.0124*	17	10		9	18	
	rs4132670	CC (222)	24.12±3.26		85.95±7.78		120.9±32.86		120	102		81	141	0.4605
		CT (101)	23.98±3.07		88.52±8.32		132.1±3.72		55	46	0.7452	44	57	
		TT (19)	24.88±2.07	0.4004	91.79±6.42	0.0007*	149.5±59.59	0.0027*	12	7		8	11	
PCSK2	**rs2269023**	CC (117)	24.30±3.41		87.65±6.85		124.8±31.73		73	44		55	62	<0.0001*
		CT (164)	23.89±3.13		85.74±8.79		120.1±33.40		67	97	<0.0001*	42	122	
		TT (61)	24.41±2.68	0.1910	89.33±7.42	0.0101*	143.1±48.24	<0.0001*	47	14		36	25	

FPG, Fasting plasma glucose; BMI, Body mass index; FHD, Family history of diabetes; PPBG, 2 hours Postprandial Blood Glucose.
p-value is calculated by Kruskal-wallis test; *p-value is significant

Table 14b: Association of selected genetic variants of selected genes from insulin synthesis pathway with clinicopathological pathway:

GENE	SNPs	Genotype	BMI		FPG (mg/dl)		PPBG (mg/dl)		Acanthosis Nigricans			FHD		
			Mean±SD	p-value	Mean±SD	p-value	Mean±SD	p-value	Yes	No	p-value	Yes	No	p-value
KCNJ11	rs5219	CC(170)	24.21±3.27	0.8048	85.88±8.18	0.0075*	125.6±41.49	0.0207*	92	78	0.4310	61	109	0.5168
		CT(135)	24.00±3.16		87.62±7.57		122.6±29.62		78	57		57	78	
		TT(37)	24.18±2.55		90.19±8.07		138.3±35.98		17	20		15	22	
ABCC8	rs757110	TT(181)	24.16±3.15	0.9565	85.76±7.79	0.0011*	123.9±40.61	0.0125*	96	85	0.8087	66	115	0.2714
		TG(144)	24.06±3.21		88.07±7.92		125.7±29.95		81	62		57	86	
		GG(17)	21.21±2.82		91.56±8.72		146.1±41.85		10	8		10	8	
MTNR1B	rs10830963	CC(168)	23.83±3.11	0.2764	85.88±7.83	0.0015*	121.5±35.40	0.0017*	86	82	0.1743	63	105	0.6820
		CG(134)	24.57±3.29		87.34±8.15		127.9±39.44		74	60		52	82	
		GG(40)	23.87±2.72		90.85±7.28		136.6±31.04		27	13		18	22	

FPG, Fasting plasma glucose; BMI, Body mass index; FHD, Family history of diabetes; PPBG, 2 hours Postprandial Blood Glucose.
p-value is calculated by Kruskal-wallis test; *p-value is significant

Placental histopathological alterations and GLUT1 & GLUT3 expression versus adverse outcome of neonates

Association study by chi-square analysis: Among the neonatal anomalies, macrosomia and neonatal hypoglycaemia were found in the newborn of GDM mother in significantly higher frequency than that of non-GDM one, and hence were selected for the association study; similarly, among the GLUTs, placental expression level of GLUT1 and GLUT3 (protein) were considered. In GDM women, significant association of placental histological abnormalities like villous edema, chorangiosis, increased syncytial knots and thickening of villous basement membrane was found with macrosomia (p=0.0001, p=0.0003, p=0.005 and p=0.01 respectively) and neonatal hypoglycaemia (p=0.0004, p=0.001, p=0.0008 and p=0.015 respectively) (Table-15a). Among the GLUTs, association of high placental GLUT1 level also remained significant with both macrosomia (p=0.0003) and neonatal hypoglycaemia (p=0.0009); however, in GLUT3, although comparatively higher expression level of the protein suggested a trend of association with the said neonatal anomalies, the strength was not statistically significant (p=0.17 and p=0.054 respectively).

Logistic regression analyses: In multivariate logistic regression analysis, placental alterations that were found to be associated significantly with macrosomia and neonatal hypoglycemia, were included as covariates and incidence of the abnormalities were considered as the outcome. Association of villous edema, chorangiosis, and placental GLUT1 protein remained significant with higher risk for both macrosomia (OR=12.29, 95%CI=1.36-110.82, p=0.025; OR=16.09, 95%CI=1.5-172.45, p=0.022**;** OR=11.47, 95%CI=1.36-96.58, p=0.025**)** and neonatal hypoglycaemia (OR=6.52, 95%CI= 1.05-40.5, p=0.044; OR=8.95, 95%CI=1.20-66.45, p=0.032; OR=6.22, 95%CI=1.010-38.33, p=0.049) (Table-15b). However increased syncytial knots were found to be associated with significantly higher risk of neonatal hypoglycemia (OR=10.74, 95%CI=1.24-93.25, p=0.031) and villous basement membrane thickening did not show any significant association with either of the neonatal anomalies.

Table 15A: Association of macrosomia and neonatal hypoglycaemia with Placental histological abnormalities and expression level of GLUT1 and GLUT3 in GDM subjects.

		Macrosomia		p value#	Neonatal Hypoglycemia		p value#
		Present (n=12)	Absent (n=58)		Present (n=13)	Absent (n=57)	
Immature Villi	Yes	2 (7%)	25 (93%)	0.1113	4 (15%)	23 (85%)	0.7534
	No	10 (23%)	33 (77%)		9 (21%)	34 (79%)	
Chorangiosis	Yes	10 (40%)	15 (60%)	**0.0003***	10 (40%)	15 (60%)	**0.001***
	No	2 (4%)	43 (96%)		3 (7%)	42 (93%)	
Villous edema	Yes	10 (43%)	13 (57%)	**0.0001***	10 (43%)	13 (57%)	**0.0004***
	No	2 (4%)	45 (96%)		3 (6%)	44 (44%)	
Fibrinoid deposition	Yes	5 (19%)	21 (79%)	0.7509	7 (27%)	19 (73%)	0.2093
	No	7 (16%)	37 (84%)		6 (14%)	38 (86%)	
Vascular Congestion	Yes	0 (0%)	5 (100%)	0.5793	0 (0%)	5 (100%)	0.5757
	No	12 (18%)	53 (82%)		13 (20%)	52 (80%)	
Increased no. of syncytial Knots	Yes	5 (55%)	4 (45%)	**0.0056***	6 (66%)	3 (34%)	**0.0008***
	No	7 (11%)	54 (89%)		7 (11%)	54 (89%)	
Thickening of BM	Yes	5 (50%)	5 (50%)	**0.0102***	5 (50%)	5 (50%)	**0.0154***
	No	7 (12%)	53 (88%)		8 (13%)	52 (87%)	
Calcification	Yes	1 (33%)	2 (67%)	0.4363	1 (33%)	2 (67%)	0.4655
	No	11 (16%)	56 (84%)		12 (18%)	55 (82%)	
NRBC	Yes	3 (33%)	6 (67%)	0.1774	3 (33%)	6 (67%)	0.3526
	No	9 (15%)	52 (85%)		10 (16%)	51 (84%)	
GLUT1	H	9 (45%)	11 (55%)	**0.0003***	9 (45%)	11 (55%)	**0.0009***
	L/Int.	3 (6%)	47 (94%)		4 (8%)	46 (92%)	
GLUT3	H	3 (33%)	6 (67%)	0.1774	4 (44%)	5 (66%)	0.0547
	L/Int.	9 (15%)	52 (85%)		9 (15%)	52 (75%)	

BM, basement membrane; NFRBC, nucleated fetal red blood cell; GLUT, glucose transporter; L, low; Int, intermediate; L, low. *p value significant; #p value calculated by Fischer‟s exact test

Table 15B: Multivariate logistic regression analysis for association study of Macrosomia and hypoglycaemia with Placental histological abnormalities and GLUT expression in GDM

	Macrosomia		Hypoglycemia	
	OR (95%CI)	p value	OR (95%CI)	p value
Chorangiosis	16.09(1.5-172.45)	0.022*	8.95(1.20-66.45)	0.032*
Villous edema	12.29(1.36-110.82)	0.025*	6.52(1.05-40.58)	0.044*
Increased no. of syncytial Knots	5.37(0.407-70.84)	0.202	10.74(1.24-93.25)	0.031*
Thickening of basement membrane	1.78(0.167-19.01)	0.632	1.54(0.177-13.46)	0.695
GLUT1	11.47(1.36-96.58)	0.025*	6.22(1.010-38.33)	0.049*

OR, Odd ratio; CI, Confidence interval; *p value significant

Discussion:

Prevalence of GDM (gestational diabetes mellitus) in our study was 17.27% in kolkata and its adjoining region and the data was in accordance to overall GDM spectrum of India [5]. In our study, higher odds ratio of risk factors like maternal age, acanthosis nigricans and family history of diabetes (FHD) for the disease was in accordance to previous report in different population [48,49,50]. However, association of pre-gestational BMI with GDM was not found besides its previous report as disease etiological factor [10]. However, our data was in accordance to Li et al (2016) and Yilmaz et al (2014) in study subjects of southern China and Turkey [51,15].

Insulin resistance was well known in GDM pathogenesis; oxidative stress and sub-clinical inflammation was considered as major contributor of insulin resistance [52,53] although remained less explored in GDM. In our study, significantly higher ROS (reactive oxygen species) and higher level of DNA damage measured by comet tail parameters in case subjects compared to control provided direct evidence for association of oxidative stress and DNA damage in pathogenesis of GDM. No studies reported these on sample set although in few increase of oxidative stress markers and occurrence of DNA damage was addressed separately in GDM [52,54]. Subclinical inflammation might be associated with GDM pathogenesis with respect to interfering effect of inflammatory cytokines on insulin sensitivity [55]. Some hematological parameters like WBC, NLR (neutrophil to lymphocyte), MPV (mean platelet volume) were found as inflammatory indicator in GDM [14,15]. In our study, significantly higher values of WBC, NLR and MPV in GDM compared to control indicated association of higher degree of inflammation with the disease condition. More over oxidative stress along with DNA damage and inflammatory indicators (WBC, NLR and MPV) were established as significant determinants of disease condition independent of pre-gestational BMI. In concordance, Frohlich et al.,2000 [56] found no association of C-reactive protein level (inflammatory markers with obesity and Pannacciulli et al.,2001 [57] postulated higher diabetic risk in women with high level of CRP independent to obesity. Thus in our study, association of oxidative stress and inflammation was established in pathogenesis of GDM; however role of obesity was not established in this respect in our studied population

During pregnancy in response to insulin resistance, pancreatic β cells undergo several changes of which enhanced insulin synthesis, processing and increased glucose stimulated insulin secretion are most important [58]; in GDM, alteration of one or more of these processes

under influence of genetic factors was considered as a major event of disease pathogenesis. In present study, association of genetic variants of insulin synthesis, processing and secretion with gestational diabetes mellitus was first time reported from an Eastern Indian population, mainly of Kolkata, West Bengal.

For insulin synthesis and processing pathway, *TCF7L2*, a candidate gene of type-2 diabetes and its three intronic variants (rs12255372, rs7903146and rs4132670) were selected. In rs12255372 and rs7903146, frequencies of risk alleles (T in both) in control individuals of our population remained comparable to that reported in NGT subject (normal glucose tolerant test) of an Asian Indian population (rs12255372, T=0.11 vs. 0.19; rs12255372, T=0.28 in both) [59]. In rs12255372, significantly higher frequency of risk allele (T)in GDM than nonGDM subjects (p=0.045) and about 1.9 fold increased disease risk in women carrying T allele than those with G (safe) allele indicated strong association of the variant in development of GDM of studied population. Our result was very much consistent with that of Papadopoulou et al.,2011[60] in GDM subjects from a Swedish population. However, in other studies, Michalak-wojnowsket et al, 2016 & de Melo et al. [61,62] did not find any association of the SNP with GDM in Polish and Euro-Brazilian populations respectively.

In rs7903146, although association of risk allele (T) with GDM did not reach to statistical significance (p=0.25) in our study, homozygous risk genotype (TT) showed significant association with disease in co-dominant (TT vs. CC; p=0.005) and recessive (TT vs. CC+TT; p=0.004) models with odds ratios of 3.46 and 3.79 respectively. Association of rs7903146 with respect to alleles and genotypes were reported in GDM by Shat et al., 2007 [63] in Southern-Swedish population, Lauenborg et al.,2008 [64] in Danish Caucasians population; on the contrary, Klein et al.,2012 & de Melo et al.,2015 [65, 62] did not find such association in Caucasian and Euro-Brazilian populations respectively. Therefore, data on association rs12255372 and rs7903146 with GDM showed wide variation and ethnicity of the studied population might be a strong influencing factor. To our best, present study explored association of rs12255372 and rs7903146 for the first time in Indian population and the result seemed to be important as association of the two SNPs was already established with type-2 diabetes mellitus (T2DM) in Indian scenario [59]. Association of rs4132670 was not explored with GDM previously in any population to our best. In Indian population rs4132670 was not studied previously; however allelic frequency of the SNP found in control population of our study was comparable to that of Estonian and North-Swedish population reported in dbSNP

website(https://www.ncbi.nlm.nih.gov›snp) (C=0.84 vs. 0.78 and 0.77; T=0.16 vs. 0.22 and 0.23 respectively). Minor allele (T) of rs4132670 seemed to be the risk allele in our population as was significantly more frequent (p=0.002) in GDM than control and associated with 2.26 fold higher disease risk in individuals carrying it than those with safe/major (C) allele. Concordantly homozygous risk genotype (TT) and heterozygous genotype (CT) showed association with significantly higher odds ratio in co-dominant, dominant and recessive models. So far in one study with GWAS data set, Zhu et al,2013 [66] predicted association of rs4132670 with T2DM, obesity or both.

In *PCSK2*, one intronic variant rs2269023 (C/T) was analyzed and minor allele (T) frequency in our control group was similar to that reported in Han Chinese population (T=0.38 versus 0.35)[66]. For rs2269023, T seemed to be the risk allele in our population as evident from its higher frequency in case compared to control (T=0.49 versus 0.38, p=0.06) and significant association of TT genotype with increased GDM risk in co-dominant and recessive models. Till date, association of rs2269023 was explored with T2D only in Han Chinese [25] and Afro-European populations; [67] our study suggested potential association of the variant with GDM in West Bengal. However, we failed to establish any association of the variant with impaired pro-insulin conversion in our population.

The functional link of *TCF7L2* with hyperglycemia at molecular level was not well explained although insulin synthesis and processing pathway was mostly highlighted. *TCF7L2* is expressed in pancreatic β-cells; over-expression of *TCF7L2* was found to interfere with insulin synthesis and processing through its negative impact on associated genes at the transcriptional level [68]. In present study, significantly higher amount of pro-insulin and higher proinsulin/C-peptide ratio in GDM compared to non-GDM control indicated impairments of pro-insulin conversion process during disease development; significant association of these parameters with homozygous risk and heterozygous genotypes of TCF7L2 variants suggested influence of the candidate (TCFL2) in the said process in our studied population. Similar to us, Dahlgreen et al.,2007, Kirchoff et al.,2008, Zheng et al.,2012 and Cropano et al.,2017[69,70,71,72] reported significant association of TCF7L2 variants with impaired pro-insulin conversion in type-2 diabetes patients of different population; however, in these studies, association of rs4132670remained unexplored. rs7903146and rs4132670is located on intron-3 and rs12255372 on intron-4 of *TCF7L2*. In pancreatic β-cells, enhancer activity of intron-3 of *TCF7L2* was reported and homozygous risk genotypes of rs7903146 and rs4132670 were shown to have significant positive impact on gene expression [73]; the data seemed to serve as a probable explanation of our finding of association of *TCF7L2* variants especially rs4132670 (as its association with GDM not reported previously) with the criteria of impaired pro-insulin conversion. Therefore, in our population, allelic variation in intronic SNPs of TCF7L2 seemed to enhance the gene expression in pancreatic islets that might interfere with insulin synthesis/processing pathway to predispose individuals for development of GDM during pregnancy.

From insulin secretion pathway, total 4 genetic variants, one each from *KCNJ11* (rs5219) and *ABCC8* (rs757110) and two from *MTNR1B* (rs1387153 and rs10830963) were selected for the present study.

Risk allele frequency of rs5219 in control subject of our population was very similar to that reported in normal individuals of an Asian Indian population (T=0.28 vs. 0.29 respectively) by Khan et al.,2015 [74]. Similarly, in rs757110, risk allele frequency in our control subject remained comparable to that found in South Indian population (G=0.22 vs. 0.17 respectively) [75]. In rs5219, risk allele frequency was not significantly higher (p=0.11) in GDM than non-GDM control, however significant higher disease odds ratio of homozygous risk genotype in co-dominant (CC vs. TT, OR=3.02, p=0.003) and recessive models (TT vs. CT+CC, OR=3.97, p=0.009) suggested probable association of the variant with GDM in the studied population. Significant association of rs757110 with increased disease risk at both allelic and genotypic level strongly indicated influence of the variant on disease development in our population. Association of rs5219remained comparatively less explored in GDM with conflicting results i.e. Shaat et al.,2005[76] reported significant association of rs5219 with GDM in Southern Swedish population; on the contrary, Popova et al.,2017 and Cho et al.,2009 [77-78] did not find such association in Russian and Korean population respectively. Association of rs757110 with GDM remained unexplored mostly and therefore our data in this respect seemed to be the first-time report of its kind to our best.

In *MTNR1B*, allelic frequency of rs1387153 in our control population was in accordance to that reported among the Indian & Asians (C=0.62 and T=0.38) [79]; In rs10830963, although minor allele frequency of our studied population (control) differs from that reported in Gujarat, India (G=0.27 vs. 0.39 respectively) [80]. Our data remained similar to the MAF (minor allele frequency) value reported for the SNP on 1000 genome (G=0.27 vs. 0.26 respectively). Allelic and genotypic distribution of rs1387153 among case and control subjects did not suggest any association of the variant with disease development in our population. However, in rs10830963, association of risk allele (G) with significantly increased disease risk and finding of similar phenomenon at genotypic level indicated strong influence of the SNP in development of GDM among pregnant women of our population. Association of rs1387153 and rs10830963 was not explored previously in eastern Indian population with any disease condition. Our data remained consistent with Vlassi et al.,2012 [81] who reported association of rs10830963 but not rs1387153 with increased risk of GDM in Greek population; however contrary to us, Kim et al.,2011[82] reported strong association of both the variants with disease in Korean population.

In our study, phenomenon of insulin resistance and impaired β-cell function was evident in GDM subjects from significantly higher values of fasting insulin, HOMA-IR, QUICKI and lower value of HOMA-β compared to control. However, we did not find any influence of rs5219, rs757110, rs1387153 and rs10830963 on fasting insulin and parameters of insulin resistance/ sensitivity (HOMA-IR/QUICKI). Similar to our finding, association of rs5219 was not found with HOMA-IR in Chinese diabetic patients [83]; in another study with African-American and Hispanic-American participants, no significant association of the SNP with insulin sensitivity index (S1) was established.[84] Similarly among MTNR1B variants, no significant influence of rs10830963 on HOMA-IR was reported in GDM subjects of a Greek population[81] and T2D subjects of Chinese population.[85] On the contrary in one study involving GDM subjects from South Indian population, association of rs5219 with insulin resistance was found.[28]

To assess pancreatic β-cell function in reference to insulin secretion HOMA-β value is widely used.[86] In present study for each of rs5219 and rs757110, individuals of homozygous risk genotypes showed reduced HOMA-β values compared to those of heterozygous and dominant homozygous genotypes; this indicated influence of the risk alleles of the variants on impaired β-cell function in our studied population. Association with β-cell function with rs5219 and rs757110 was studied in mainly in T2D subjects and among these our data was in line with the finding of significant association of rs5219 with HOMA-β value in Russian [87] and Indonesian populations [88]. Actually due to allelic variation at rs5219 (C to T) and rs757110 (T to G), substitution of glutamate occurs by lysine in 23rd position of KCNJ11 (E23K) and serine by alanine at 1369th position of ABCC8 (S1369A) respectively of K^+ATP channel.[75, 89] Human K^+ATP channel with K23 and A1369 showed reduced ATP sensitivity compared to that with E23 and S1369 in patch clamp study.[90] Actually ATP mediated closure of K^+ATP channel is an essential step towards release of insulin from pancreatic β-cells to and therefore reduced ATP sensitivity of the channel might affect the functional efficiency of β-cells.[91] Therefore in our studied population, individuals having K23 in KCNJ11 (T/ rs5219) and A1369 in ABCC8 (G/rs757110) might have impaired β-cell function as evident from reduced HOMA-β value and thus become more prone to develop GDM under conditions of pregnancy.

However for rs1387153 and rs10830963, the genetic variants of *MTNR1B*, comparable HOMA-β values among carriers of different genotypes in our study subjects provided contradictory evidence to the finding of Prokopenko et al, 2009[92] and Wang et al 2019.[85] *MTNR1B* encodes melatonin receptor that is expressed on human pancreatic β-cells. The risk allele G of intronic variant rs10830963 was shown to enhance *MTNR1B* expression and association of the phenomenon with reduced insulin secretion was reported.[93] Although association of rs10830963 was found to be significant with GDM in our study population, functional link of the risk allele was not established with the parameter of insulin secretion (HOMA- β). Similar to our finding, no significant association of rs10830963 was reported with pancreatic β-cell function in T2D patients of Indian Sikh population.[94] Like T2D and GDM, phenomenon of insulin resistance and β-cell dysfunction are frequent in polycystic ovarian syndrome (PCOS).[95] Our finding was also in full accordance to another study that reported significant association of rs10830963 with PCOS of Chinese population, but failed to establish any link of the SNP with parameter of impaired insulin secretion.[96] Therefore, our data suggested association of *MTNR1B* variants in special reference to rs10830963 with GDM in the studied population from West Bengal, India; however further study in detail is needed to explore the functional link of the variant to pathogenesis of the disease condition.

In response to diabetic insult of maternal environment, placental changes at histological and molecular level were reported and predicted to play vital role in adverse fetal/neonatal outcome. Although such studies are reported in diabetic pregnancy [97, 98, 99], comprehensive analysis on placental changes and its impact on fetal/neonatal abnormalities is relatively rare in GDM [31,100]. In the present case control study, among 11 histological abnormalities detected successfully, incidence of Chorangiosis, villous edema, thickening of basement membrane and increased syncytial knot was significantly higher in GDM placenta than that of non- GDM one. Like us, Bhattacharjee et al.,2017 and Edu et al.,2016 [101,100] reported higher incidence of similar abnormalities in GDM placental tissue than that of normal one; however sample size of case and control subjects of these studies remained comparatively low. Thickening of basement membrane of syncytiotrophoblast along with increased syncytial knots and accumulation of intra-villous fluid in villous edem aseemed to interfere with blood gas exchange between mother and fetus; these phenomenon were considered as potential cause of chronic fetal hypoxia [102,103] that might result in vascular pathological changes in placenta like chorangiosis[31]. Therefore, in present study, higher frequency of aforesaid histological abnormalities in GDM placenta than normal one confirmed incidence of higher degree of fetal hypoxia in GDM pregnancy compared to that of normal one.

Among the nutrients transported from maternal to fetal circulation across placenta, glucose seemed to be most important due to very low capacity of fetus to produce it endogenously [104]. During pregnancy under diabetic condition, high glucose concentration in maternal blood and over-expression of placental glucose transporters (GLUTs) in response seemed to alter transplacental glucose transport significantly [105, 106] that remained poorly explored in GDM with respect to cause and consequence. In present study expression of GLUT1, GLUT3, GLUT4 and GLUT8 was detected (mRNA) in term placental tissue of women from an Eastern Indian population. Although some studies reported placental expression of GLUT9 [38] and GLUT12 [107] the candidates were not found in our studied samples. In present study, significantly higher expression of GLUT1 and GLUT3 (mRNA and protein) in GDM placenta than normal one indicated their importance in enhanced fetal glucose transport under diabetic condition. Similarly with respect to overall expression level in syncytiotrophoblast, Stanirowski et al., 2017[38] reported significantly higher expression of GLUT1 in GDM placenta and Reza-Lopez et al.,2014 [108] found higher GLUT3 expression in placenta from macrosomic infants. In response to hypoxia, over-expression of GLUT1 and GLUT3 at mRNA and protein levels were reported in human placental trophoblast cells [109]. Fetal hypoxia was evident in our studied samples from placental histological abnormalities and the phenomenon seemed to up-regulate expression of GLUT1 and GLUT3 as a consequence. Among adverse neonatal outcome, macrosomia and neonatal hypoglycaemia was significantly higher in GDM pregnancy than normal one in our population. Significant association of both macrosomia and neonatal hypoglycemia with placental histological abnormalities and GLUT1 expression level and GLUT3 expression for macrosomia only suggested a role of fetal hypoxia and enhanced glucose transport in pathogenesis of these outcomes. Association of macrosomia with enhanced fetal glucose transport was reported [110]; higher glucose seemed to cause fetal hyperinsulinemia [111] that might lead to neonatal hypoglycemia after parturition. Thus in present study, histological and molecular alteration of placenta indicated incidence of fetal hypoxia and enhanced fetal glucose transport mainly through GLUT1 and GLUT3 under diabetic condition; moreover, these phenomena might have functional association with each other and have significant influence on adverse neonatal outcome.

Reference:

1. American Diabetes Association. Gestational Diabetes Mellitus (Position Statement). Diabetes Care 2004; Vol. 27 (Suppl 2), pp. S88–S90.

2. Kalra P, Kachhwaha CP, Singh HV, Prevalence of gestational diabetes mellitus and its outcome in western Rajasthan. Ind J Endocrinol and Metabolism 2013; Vol. 17, pp. 677–680.

3. Watanabe RM. Inherited destiny? Genetics and gestational diabetes mellitus. Genome Med 2011; Vol. 3, No. 18.

4. Joshi SR, Parikh RM, India - diabetes capital of the world: now heading towards hypertension. J Assoc Physicians India 2007; Vol. 55, pp. 323–324.

5. Seshiah V, Balaji V, Balaji MS et al., Prevalence of gestational diabetes mellitus in South India (Tamil Nadu) – A community based study. J Assoc Physicians India 2008; Vol. 56, pp. 329–333.

6. Sreekanthan K, Belicita A, Rajendran K et al., Prevalence of gestational Diabetes Mellitus in a Medical College in South India: A pilot study. Ind J ClinPrac 2014; Vol. 25, pp. 342–347.

7. Buchanan TA. Pancreatic B-cell defects in gestational diabetes: implications for the pathogenesis and prevention of type 2 diabetes. J ClinEndocrinol Metab, 2001; Vol. 86, pp. 989 –993.

8. Ruth J.F. Loos, Paul W. Franks, Richard W. Francis et al., TCF7L2 Polymorphisms Modulate Proinsulin Levels and β-Cell Function in a British Europid Population. DIABETES, 2007; Vol. 56, pp. 1943-1947.

9. Gloyn AL, Siddiqui J, Ellard S. Mutations in the genes encoding the pancreatic beta-cell KATP channel subunits Kir6.2 (KCNJ11) and SUR1 (ABCC8) in diabetes mellitus and hyperinsulinism. Hum Mutat 2006; Vol. 27, pp. 220 –231.

10. Vrachnis N, Belitsos P, Sifakis S et al., Role of adipokines and other inflammatory mediators in gestational diabetes mellitus and previous gestational diabetes mellitus. Int J Endocrinol 2012; Vol. 2012, 549-748.

11. Ceriello A, Motz E. Is oxidative stress the pathogenic mechanism underlying insulin resistance, diabetes, and cardiovascular disease? The common soil hypothesis revisited. ArteriosclerThrombVascBiol 2004; Vol. 24, pp. 816–823.

12. Maritim C, Sanders RA, Watkins JB. Diabetes, oxidative stress, and antioxidants: A review. J BiochemMolToxicol 2003; Vol. 17, pp. 24–38.

13. Pitozzi V, Giovannelli L, Bardini G et al., Oxidative DNA damage in peripheral blood cells in type-2 diabetes mellitus: Higher vulnerability of polymorpho nuclear leukocytes. Mutat Res 2003; Vol. 28, pp. 129–133.

14. Wolf M, Sauk J, Shah A et al., Inflammation and Glucose Intolerance: A prospective study of gestational diabetes mellitus. Diabetes care, 2004; Vol. 27, pp. 21–27.

15. Yilmaz H, Celik HT, Namuslu M et al., Benefits of the neutrophil-tolymphocyte ratio for the prediction of gestational diabetes mellitus in pregnant women. ExpClinEndocrinol Diabetes 2014; Vol. 122, pp. 39-43.

16. Vrachnis N, Belitsos P, Sifakis S et al., Role of adipokines and other inflammatory mediators in gestational diabetes mellitus and previous gestational diabetes mellitus. Int J Endocrinol 2012; Vol. 2012, pp. 549-748.

17. Founds SA, Powers RW, Patrick TE et al., A comparison of circulating TNF-alpha in obese and lean women with and without preeclampsia. Hypertens Pregnancy, 2008; Vol. 27, pp. 39–48.

18. Rota S, Yildirim B, Kaleli B et al., C-reactive protein levels in non-obese pregnant women with gestational diabetes. Tohoku J Exp Med 2005; Vol. 206, pp. 341–345.

19. Richard M. Watanabe, Mary Helen Black, Anny H. Xiang et al., Genetics of Gestational Diabetes Mellitus and Type 2 Diabetes. Diabetes Care, 2007; Vol. 30 (Suppl 2), pp. S134–S140.

20. Melani F, Arthur H. Rubenstein, Philip E. Oyer et al., Identification of Proinsulin and C-Peptide in Human Serum by a Specific Immunoassay. Proceedings of the National Academy of Sciences 1970; Vol. 67, No. 1, pp. 148-155.

21. Galloway JA, Hooper SA, Spradlin CT et. al., Diabetes mellitus, a fundamental and clinical text; Diabetes Care, 1992; Vol. 15, No. 5, pp. 666-692.

22. Zhou Y, Park SY, Su J et al., TCF7L2 is a master production of insulin production and processing. Hum Mol Genet, 2014; Vol. 15, No. 23, pp. 6419-31.

23. Shokouhi S, Delpisheh A, Haghani K. et al., Association of rs7903146, rs12255372, and rs290487 polymorphisms in TCF7L2 gene with type 2 diabetes in an Iranian Kurdish ethnic group. Clinical Laboratory, 2014; Vol. 60, No. 8, pp. 1269-76.

24. Pang D.X, Smith A.J.P., Humphries S.E., Functional analysis of TCF7L2 genetic variants associated with type 2 diabetes. Nutrition metabolism & cardio vascular disease, 2013; Vol. 23, No. 6, pp. 550-556.

25. Chang TJ, Chiu YF, Sheu WH et al. Genetic polymorphisms of PCSK2 are associated with glucose homeostasis and progression to type 2 diabetes in a Chinese population Sci Rep 2015;26(5):14380.

26. Zhang Y, Sun C. M, Hu X. Q, Relationship between melatonin receptor 1B and insulin receptor substrate 1 polymorphisms with gestational diabetes mellitus: a systematic review and meta-analysis. Scientific report, 2014; Vol. 4, No. 6113, pp. 1-7.

27. Liu Q, Huang Z, Li Het al. Arch Relationship between melatonin receptor 1B (rs10830963 and rs1387153) with gestational diabetes mellitus: a case-control study and meta-analysis. Gynecol Obstet, 2016; Vol. 294, No.1, pp. 55-61.

28. Lenin M,RamasamyR, KulkaraniS et al.,Association of KCNJ11(RS5219) gene polymorphism with biochemical markers of glycemic status and insulin resistance in gestational diabetes mellitus. Meta Gene, 2018; Vol.16, pp. 134-138.

29. Hugo V, Niki B, Debbie J, Clinical Care/Education/Nutrition, The Impact of Glycemic Control on Neonatal Outcome in Singleton Pregnancies Complicated by Gestational Diabetes. Diabetes Care, 2007; Vol. 30, No. 3.

30. Jarmuzek P, Wielgos M, Dorota A. et al., Placental pathologic changes in gestational diabetes mellitus. NeuroendocrinolLett, 2015; Vol. 36, No. 2; pp. 101–105.

31. Schoots M. H, Gordijn S. J, Scherjon S.A. et al., Oxidative stress in placental pathology. Placenta, 2018; Vol. 69, pp.153e161.

32. Daskalakis G, Marinopoulos S, Krielesi V, Placental pathology in women with gestational diabetes. ActaObstetriciaetGynecologica, 2008; Vol. 87, pp.403-407.

33. Rudge M.V.C, Lima C.P, Damasceno D. C et al., Histopathological placental lesions in mild gestational hyperglycemic and diabetic women. Diabetology& Metabolic Syndrome, 2011; Vol.3, No.19.

34. Aldoretta P.W, Carver T.D, Hay W.W, Ovine uteroplacental glucose and oxygen metabolism in relation to chronic changes in maternal and fetal glucose concentrations. Placenta, 1994; Vol. 15, No. 7, pp. 753-764.

35. Kainulainen H, Jarvinen T, Heinonen P.K, Placental glucose transporters in fetal intrauterine growth retardation and macrosomia. GynecolObstet Invest, 1997; Vol 44, pp. 89-92.

36. Brown K, Heller DS, Zamudio S, Illsley N., Glucose transporter 3 (GLUT3) proteinexpression in human placenta across gestation. Placenta, 2011; Vol 32, pp. 1041-9.

37. Acevedo GC, Marquez JL, Rojas S et al., Insulin and nitric oxide stimulates glucose transport in human placenta. Life Sci, 2005, Vol. 76, pp.2643–53.

38. Stanirowski PJ, Szukiewicz D, Pyzlak M, et al., Impact of pre-gestational and gestational diabetes mellitus on the expression of glucose transporters GLUT-1,GLUT-4 and GLUT-9 in human term placenta. Endocrine, 2017; Vol. 55, pp.799–808.

39. Acosta O, Ramirez VI, Lager S, et al., Increased glucose and placental GLUT-1 in large infants of obese nondiabetic mothers. Am J ObstetGynecol, 2015; Vol 7, pp. 212:227.

40. Seshiah V, Das A, Balaji V et al., Gestational Diabets Mellitus Guidelines. J Assoc Physicians India 2006;34: 622-628.
41. Manea A., Constantinescu E., PopovD., Changes in oxidative balance in rat pericytes exposed to diabetic conditions. Journal of Cellular and Molecular Medicine, 2004; Vol.8, No.1, pp.117-126.
42. Sambrook J, Fritsch EF, Maniatis T., Molecular cloning: a laboratory manual, 2nd edn. Cold Spring Harbor. NY: Cold Spring Harbor Laboratory, 1989.
43. Endo S, Maeda K, Suto M et al. Differences in Insulin Sensitivity in Pregnant Women with Overweight and Gestational Diabetes Mellitus. Gynecol Endocrinol 2006;22(6):343-349.
44. Barber R D, Harmer D W, Coleman R A et al., GAPDH as a housekeeping gene: analysis of GAPDH mRNA expression in a panel of 72 human tissues. Physiol Genomics, 2005; Vol. 11, No. 21(3), pp. 389-95.
45. Livak K. J and Schmittgen T. D. Analysis of Relative Gene Expression Data Using RealTime Quantitative PCR and the 22DDCT Method. Methods, 2001; Vol. 25, pp. 402–408.
46. Hahn T, Barth S, Graf R. Placental glucose transporter expression is regulated by glucocorticoids. J Clin Endocrinol Metab, 1999; Vol. 84, No. 4, pp. 1445-52.
47. Perrone F, Suardi S, Pastore E et al., Molecular and cytogenetic subgroups of oropharyngeal squamous cell carcinoma. Clin Cancer Res, 2006; Vol. 12, pp.6643–6651.
48. Lao TT, Ho LF, Chan BC et al. Maternal age and prevalence of gestational diabetes mellitus. Diabetes Care, 2006; Vol. 29, pp. 948–949
49. Retnakaran R, Connelly PW, Sermer M et al., The impact of family history of diabetes on risk factors for gestational diabetes. Clin Endocrinol , 2007; Vol. 67, pp. 754–760.
50. Higgins SP, Freemark M, Prose NS. Acanthosis nigricans: A practical approach to evaluation and management. Dermatol Online J, 2008; Vol. 14, No. 2.
51. Li P, Yin Y, Lin S et al. Utility of pregestational body mass index and initial fasting plasma glucose in predicting gestational diabetes mellitus. Am J Med Sci, 2016; Vol.

351, pp. 420–425.

52. Zhu C, Yang H, Geng Q et al. Association of oxidative stress biomarkers with gestational diabetes mellitus in pregnant women: A case-control study. PLoS One, 2015; Vol. 10, pp. e0126490.

53. Richardson AC, Carpenter MW et.al., Inflammatory mediators in gestational diabetes mellitus. Obstet Gynecol Clin North Am, 2007; Vol. 34, pp. 213–224.

54. Gelaleti RB, Damasceno DC, Lima PH et al. Oxidative DNA damage in diabetic and mild gestational hyperglycemic pregnant women. Diabetol Metab Syndr, 2015; Vol.7, No.1.

55. Winzer C, Wagner O, Festa A et al. Plasma adiponectin, insulin sensitivity, and subclinical inflammation in women with prior gestational diabetes mellitus. Diabetes Care, 2004; Vol. 27, pp. 1721–1727.

56. Fröhlich M, Imhof A, Berg G et al. Association between C-reactive protein and features of the metabolic syndrome: a population-based study. Diabetes Care, 2000; Vol. 23, pp. 1835–1839.

57. Pannacciulli N, Cantatore FP, Minenna A et al. C-reactive protein is independently associated with total body fat, and insulin resistance in adult women. Int J Obes Relat Metab Disord ,2001; Vol. 25, pp. 1416–1420.

58. Sorenson R.L, Brelje T.C, Adaptation of islets of Langerhans to pregnancy: beta-cell growth, enhanced insulin secretion and the role of lactogenic hormones. HormMetab Res, 1997; Vol. 29, pp.301–307.

59. Bodhini D, Radha V, Dhar M et al., The rs12255372(G/T) and rs7903146(C/T) polymorphisms of the TCF7L2 gene are associated with type 2 diabetes mellitus in Asian Indians. Metabolism, 2007; Vol. 56, No. 9, pp.1174-8.

60. Papadopoulou A, Lynch KF, Shaat N et al., Gestational diabetes mellitus is associated with TCF7L2 gene polymorphisms independent of HLA-DQB1 *0602 genotypes and islet cell autoantibodies. Diabet Med., 2011, Vol.28, No. 9, pp.1018-27.

61. Michalak W M, Gorczyca S D, Gorczyca T et al., Association between rs7901695 and rs7903146 polymorphisms of the TCF7L2 gene and gestational diabetes in the population of Southern Poland. Ginekol Pol, 2016; Vol. 87, No. 11, pp. 745-750.

62. de Melo S. F, Frigeri H R, dos Santos W I C et al., Polymorphisms in FTO and TCF7L2 genes of Euro Brazilian women with gestational diabetes. Clin Biochem, 2015; Vol. 48, No. 16-17, pp. 1064-7.

63. Shaat N, Lernmark A, Karlsson E et al, A variant in the transcription factor 7-like 2 (TCF7L2) gene is associated with an increased risk of gestational diabetes mellitus. Diabetologia. 2007; Vol. 50, No.59, pp. 72-9.

64. Lauenborg J, Grarup N, Damm P et al., Common type 2 diabetes risk gene variants associate with gestational diabetes. J Clin Endocrinol Metab, 2009; Vol. 94, No. 1, pp. 145-50.

65. Klein K, Haslinger P, Bancher-T. D et al, Transcription factor 7-like 2 gene polymorphisms and gestational diabetes mellitus.Journal of Maternal Fetal Neonatal Medicine, 2012; Vol. 25, No.9, pp. 1783-6.

66. Zhu Z, Tong X,Liang Met al, Development of GMDR-GPU for gene-gene interaction analysis and its application to WTCCC GWAS data for type 2 diabetes. PLoS One, 2013; Vol. 8, No. 4, pp. e61943.

67. Leak TS, Keene KL, Langefeld CD et al. Association of the proprotein convertase subtilisin/ kexin-type 2 (PCSK2) gene with type 2 diabetes in an African American population. Mol Genet Metab 2007;92(1-2):145-150.

68. Zhou Y, Park S Y, Su J et al.,a master regulator of insulin production and processing. Hum Mol Genet, 2014; Vol. 15, No.23, pp. 6419-31.

69. Dahlgren A, Zethelius B, Jensevik K et al. Variant of the TCF7L2 gene are associated with beta cell dysfunction and confer an increased risk of type 2 diabetes mellitus in the ULSAM cohort of Swedish elderly men. Diabetologia, 2007; Vol. 50, No. 9, pp. 1852.

70. Kirchhoff K, Machicao F, Haupt A et al., Polymorphisms in the TCF7L2, CDKAL1 and SLC30A8 genes are associatedwith impaired proinsulin conversion.Diabetologia, 2008, Vol. 51, No.4; pp. 597-601.

71. Zheng X, Ren W, Zhang S et al., Association of type 2 diabetes susceptibility genes (TCF7L2, SLC30A8, PCSK1 and PCSK2) and proinsulin conversion in a Chinese population. MolBiol Rep, 2012; Vol. 39, No.1, pp.17-23.

72. Cropano C, Santoro N, Groop L et al, The rs7903146 Variant in the TCF7L2 Gene Increases the Risk of Prediabetes/Type 2 Diabetes in Obese Adolescents by Impairing β-Cell Function and Hepatic Insulin Sensitivity. Diabetes Care, 2017; Vol.40, No.8, pp. 1082-1089.

73. Pang D X, Smith A J, Humphries S E, Functional analysis of TCF7L2 genetic variants associated with type 2 diabetes, 2013; Vol. 23, No. 6, pp. 550-6

74. Khan I A, Vattam K K, Jahan P et al., Correlation between *KCNQ1* and *KCNJ11* gene polymorphisms and type 2 and post-transplant diabetes mellitus in the Asian Indian population. Genes Dis., 2015; Vol. 2, No. 3, pp. 276-282.

75. Viji. D, Aswathi P, Pricilla Charmine .P, Genetic Association of *ABCC8 rs757110* polymorphism with Type 2 Diabetes Mellitus risk:A case-control study in South India and A Meta-analysis. Genrep, 2018; Vol.10.

76. Shaat N, Ekelund M, Lernmark A et al, Association of the E23K polymorphism in the KCNJ11 gene with gestational diabetes mellitus. Diabetologia, 2005; Vol. 48, No.12, pp. 2544-51.

77. Popova PV, Klyushina AA, Vasilyeva LB et al., Effect of gene-lifestyle interaction on gestational diabetes risk. Oncotarget, 2017; Vol. 8, No. 67, pp. 112024-112035.

78. Cho YM, Kim TH, Lim S et al, Type 2 diabetes-associated genetic variants discovered in the recent genome-wide association studies are related to gestational diabetes mellitus in the Korean population.Diabetologia, 2009; Vol. 52, No. 2, pp. 253-61.

79. Chambers JC[1], Zhang W, Zabaneh D et al., Common genetic variation near melatonin receptor MTNR1B contributes to raised plasma glucose and increased risk of type 2 diabetes among Indian, Asians and European Caucasians; Diabetes.,2009, Vol.58, No.11, pp. 2703-8.

80. Patel R, Rathwa N, Palit et al., Association of melatonin & *MTNR1B* variants with type 2 diabetes in Gujarat population. Biomedicine & Pharmacotherapy,2018; Vol. 103,pp.429-34.

81. Vlassi M, Gazouli M, Paltoglou G et al., The rs10830963 variant of melatonin

receptor MTNR1B is associated with increased risk for gestational diabetes mellitus in a Greek population. Hormones (Athens), 2012; Vol.11, No. 1, pp.70-6.

82. Kim JY, Cheong HS, Park BL, et al, Melatonin receptor 1B polymorphisms associated with the risk ofgestational diabetes mellitus. BMC Med Genet, 2011; Vol.12, pp. 82.

83. He Y-y, Zhang R, Shao X-y, Hu C, Wang C-r, Lu J-x, Bao Y-q, Jia W-p, Xiang K-s. Association of KCNJ11 and ABCC8 genetic polymorphisms with response to repaglinide in Chinese diabetic patients. Acta Pharmacol Sin 2008;29(8):983-989.

84. Palmer ND, Langefeld CD, Bryer-Ash M, Rotter JI, Taylor KD, Bowden DW. Association of the Kir6.2 E23K Variant with Reduced Acute Insulin Response in African-Americans J Clin Endocrinol Metab 2008, 93(12):4979–4983.

85. Wang T, Wang X-t, Lai R, Ling H-w, Zhang F, Lu Q, Lv D-m, Yin X-x. *MTNR1B* Gene Polymorphisms Are Associated With the Therapeutic Responses to Repaglinide in Chinese Patients With Type 2 Diabetes Mellitus Front Pharmacol. 2019; 10: 1318.

86. Song Y, Manson JE, Tinker L, Howard BV, Kuller LH, Nathan L, Rifai N, Liu S. Insulin Sensitivity and Insulin Secretion Determined by Homeostasis Model Assessment (HOMA) and Risk of Diabetes in a Multiethnic Cohort of Women: The Women's Health Initiative Observational Study. Diabetes Care 2007; 30(7): 1747–1752.

87. Nikitin AG, Potapov VY, Brovkina OI, Koksharova EO, Khodyrev DS, Philippov YI, Michurova MS, Shamkhalova MS, Vikulova OK, Smetanina SA, Suplotova LA, Kononenko IV, Kalashnikov VY, Smirnova OM, Mayorov AY, Nosikov VV, Averyanov AV, Shestakova MVAssociation of polymorphic markers of genes FTO, KCNJ11, CDKAL1, SLC30A8, and CDKN2B with type 2 diabetes mellitus in the Russian population. PeerJ 2017; 5:e3414.

88. Sunita R, Sadewa AH, Farmawati A. Lower HOMA-b values are detected among individuals with variant of E23K polymorphism of potassium inwardly-rectifying channel subfamily J, member 11 (KCNJ11) gene. The Egyptian Journal of Medical Human Genetics 2015; 16: 227–231.

89. Souza SW, Alcazar LP, Arakaki PA, Santos-Weiss ICR, Alberton D, Picheth G, Rego FGM. Polymorphism E23K (rs5219) in the KCNJ11 Gene in Euro-Brazilian Subjects With Type 1 and 2 Diabetes. Genet Mol Res 2017;16(2).

90. Hamming KSC, Soliman D, Matemisz LC, Niazi O, Lang Y, Gloyn AL, Light PE. Co-expression of the Type 2 diabetes Susceptibility Gene Variants KCNJ11 E23K and ABCC8 S1369A Alter the ATP and Sulfonylurea Sensitivities of the ATP-Sensitive K+ Channel. Diabetes 2009; 58(10): 2419–2424.

91. Koster JC, Permutt MA, Nichols CG. Diabetes and insulin secretion: the ATP-sensitive K+ channel (K ATP) connection. Diabetes 2005;54(11):3065-3072.

92. Prokopenko I, Langenberg C, Florez JC, et al. Variants in MTNR1B influence fasting glucose levels. Nat Genet 2009; 41: 77-81.

93. Lyssenko V, Nagorny C L, Erdos M R et al., Common variant in *MTNR1B* associated with increased risk of type 2 diabetes and impaired early insulin secretion, Nat Genet, 2009; Vol. 41, No. 1, pp. 82-8.

94. Been LF, Hatfield JL, Shankar A, Aston CE, Ralhan S, Wander GS, Mehra NK, Singh JR, Mulvihill JJ, Sanghera DK. A low frequency variant within the GWAS locus of MTNR1B affects fasting glucose concentrations: genetic risk is modulated by obesity. Nutr Metab Cardiovasc Dis 2012; 22(11):944-951.

95. Macut D, Bjekić-Macut J, Rahelić D, Doknić M. Insulin and the polycystic ovary syndrome. .Diabetes Res Clin Pract 2017;130:163-170.

96. Xu X-H, Kou L-C, Wang H-M, Bo C-M, Song X-C. Genetic polymorphisms of melatonin receptors 1A and 1B may result in disordered lipid metabolism in obese patients with polycystic ovary syndrome. Mol Med Rep 2019;19(3):2220-2230.

97. Tewari V, Tewari A, Bhardwaj N et al.,Histological and histochemical changes in placenta of diabetic pregnant females and its comparision with normal placenta. Asian Pacific Journal of Tropical Disease,2011; Vol.1, pp. 1-4.

98. Dubova EA, Pavlov KA, Yesayan RM, et al., Morphometric characteristics of placental villi in pregnant women with diabetes.Bull ExpBiol Med,2011; Vol.151, No. 5, pp. 650-4.

99. Jirkovská M, Kučera T, Dvořáková V et al.,Impact of maternal diabetes type 1 on proliferative potential, differentiation and apoptotic activity in villous capillaries of term placenta.Placenta, 2016; Vol. 40, pp. 1-7.

100. Edu A, Teodorescu C, Dobjanschi CG, Socol ZZ, Teodorescu V, Matei A, Albu DF, Radulian G. Placenta changes in pregnancy with gestational diabetes. Rom J Morphol Embryol. 2016;57(2):507-12.

101. Bhattacharjee D, Mondal S K, Garain P et al., Histopathological study with immunohistochemical expression of vascular endothelial growth factor in placentas of hyperglycaemic and diabetic women. J Lab Physicians, 2017; Vol. 9, No. 4, pp. 227-233.

102. Kovalovszki L, Villányi E, Benkó G, et al., Placental villous edema: a possible cause of antenatal hypoxia. ActaPaediatr Hung, 1990; Vol. 30, No. 2, pp. 209-15.

103. Pardi G, Marconi AM, Cetin I,et al., Pathophysiology of intrauterinegrowth retardation: role of the placenta. ActaPaediatr Suppl, 1997; Vol 423, pp. 170-2.

104. Marconi AM, Cetin I, Davoli E, et al., Anevaluation of fetalglucogenesis in intrauterine growth-retarded pregnancies.Metabolism,1993; Vol.42, pp.860-4.

105. Hauguel S, Desmaizieres V, Challier JC, et al. Glucose uptake, utilization and transferby the human placenta as functions of maternal glucose concentration. PediatrRes, 1986; Vol. 20, pp. 269–73.

106. Jansson T, Wennergren M, Illsley NP et al., Glucose transporter expression and distribution in the human placenta throughout gestation and in intrauterine growthretardation. J ClinEndocrinol Metab, 1993; Vol.77, No. 1554–62.

107. Gude NM, Stevenson JL, Murthi P. et al,Expression of GLUT12 in the fetal membranes of the human placenta. Placenta, 2005; Vol. 26, No.1, pp. 67-72.

108. Reza-López SA, Sánchez-Ramírez BE, Gutiérrez-Torres DS, et al.,Expression of glucose transporters in placenta from macrosomic infants. J Med Med Sci, 2014; Vol.3, No.10, pp. 315-321.

109. Esterman A, Greco M A, Mitani Y et al., The effect of hypoxia on human trophoblast in culture: morphology, glucose transport and metabolism. Placenta, 1997; Vol.18, No. 2-

3, pp. 129-36.

110. Baumann MU, Deborde S, Illsley NP et al., Placental glucose transfer and fetal growth. Endocrine, 2002; Vol.19, pp. 13–22.

111. Olmos PR, Borzone GR, Poblete A et al., Gestational Diabetes: Glycemic Control in the Last Two Weeks Before Delivery Contributes to Newborn Insulinemia.J ObstetGynaecol Can, 2018; Vol. 40, No. 11, pp. 1445-1452.

ABBREVIATION

GDM= Gestational diabetes mellitus.

DIPSI= Diabetes in Pregnancy Study Group India.

n= total number of samples.

0C= Degree Celsius.

bp= Base pairs.

BMI= Body mass index.

OR= Odds ratio.

CI= Confidence interval.

SD= Standard deviation.

ROS= reactive oxygen species.

AFU= arbitary fluorescent unit.

WBC= white blood cell.

RBC= red blood cell.

PLT= platelets.

PDW= red blood cells volume distribution width.

NLR= neutrophil–lymphocyte ratio.

MPV= mean platelet volume.

MCV= mean cell volume.

HGB= hemoglobin.

HCT= hematocrit.

MCH= mean cell hemoglobin.

MCHC= mean corpuscular hemoglobin.

B #= Non-standardized coefficient.

β $= Standardized coefficient.

OGTT= oral glucose tolerance test.

FPG= Fasting plasma glucose.

PPBG= Postprandial Blood Glucose.

NICU= newborn intensive care unit.

NRBC= Nucleated red blood cells.

CON= Control group.

HbA1C= Glycosylated haemoglobin

GLUT1= Glucose transporter 1.

GLUT3= Glucose transporter 3.

Institute of Post Graduate Medical Education & Research
244, A.J.C. Bose Road, Kolkata – 700020.

IPGME&R Research Oversight Committee

(Institutional Ethics Committee)

Memo No. Inst/IEC/2015/544B

Date: 10.12.2015

Dr. Amlan Ghosh
Assistant Professor
Department of Biological Sciences
Presidency University, Kolkata

Dear Dr. Ghosh,

A meeting of the Institutional Ethics Committee of IPGME&R, Kolkata, was held on 05.12.2015 at 12:00 Noon in the IPGME&R Dean's Office. In this meeting, the members considered the protocol of your project titled:

Identification of molecular marker associated with the development and pathogenesis of gestational diabetes mellitus in West Bengal, India.

The following additional documents were reviewed:

- Informed consent documents in English.
- Informed consent documents in Hindi.
- Informed consent documents in Bengali.

After deliberations and review the committee took the following decision regarding the above documents:

Approved

You may therefore go along with the project in its present form. It is placed on record that the decision regarding your proposal was unanimous and therefore did not require any voting procedure.

The committee understands that from IPGME&R, Kolkata, the Co-Investigators for your project would be Dr. Subhankar Chowdhury, Professor & Head, Dept. of Endocrinology, Dr. Chhanda Datta, Professor, Dept. of Pathology and Dr. Debasmita Mandal, Associate Professor, Dept. of Gynecology & Obstetrics.

The list of committee members present in this meeting are appended to this page.

Additional points, if any, stated on the next page are to be noted.

Continued on Page 2

Jayita Basu.
05/11/19.

Institute of Post Graduate Medical Education & Research
244, A.J.C. Bose Road, Kolkata – 700020.

IPGME&R Research Oversight Committee

(Institutional Ethics Committee)

Continued from Page 1

Additional points to be noted

- The Committee expects that any amendments to the Study Protocol, Informed Consent documents or other relevant documents would be brought to its notice.
- A brief project completion report is to be submitted to the IPGME&R Research Oversight Committee. If project duration exceeds 1 year from commencement, a brief annual progress report should also be submitted.
- IPGME&R Research Oversight Committee is registered with Central Drugs Standard Control Organization (CDSCO), Government of India, in consonance with Rule 122D of the revised Drugs & Cosmetics Rules 1945 – Registration No. ECR/35/Inst/WB/2013. It functions in accordance with revised Schedule Y and Indian Council of Medical research (ICMR) guidelines.

List of institutional ethics committee members who attended the meeting on 05.12.2015

SN	Name & role in the committee	Gender	Designation
1	**Dr. Hemanta Kumar Majumder** [Scientist & Chairperson]	Male	Senior Scientist, Indian Institute of Chemical Biology, Kolkata
2	**Prof. Manju Banerjee *** [Clinician]	Female	Director, IPGME&R and Professor of Surgery
3	**Prof. Swati Chakraborty** [Clinician – Pediatrician]	Female	Consultant, Institute of Child Health, Kolkata
4	**Prof. Jayanta Chatterjee** [Clinician]	Male	Head, Department of Nuclear Medicine, IPGME&R
5	**Prof. Bijay Kumar Majumdar** [Clinician]	Male	Head, Department of Plastic Surgery, IPGME&R
6	**Prof. Amal Santra** [Basic medical scientist]	Male	Scientist, Department of Gastroenterology, IPGME&R
7	**Prof. Rajendra Pandey** [Clinician]	Male	Head, Department of Nephrology, IPGME&R
8	**Dr. Bobby Pal** [Public health expert]	Female	Assistant Professor, Department of Preventive & Social Medicine, All India Institute of Hygiene & Public Health, Kolkata
9	**Mr. Debdut Mukherjee** [Legal expert]	Male	Advocate, Calcutta High Court
10	**Mr. Arunangshu Shekhar Jana** [Social worker]	Male	Social worker, Mahendraganj, Dist South 24 Parganas
11	**Mr. Swapan Kumar Sarkar** [Lay person]	Male	Stenotypist, Office of Dean of Student Affairs, IPGME&R
12	**Prof. Avijit Hazra** [Pharmacologist & Member secretary]	Male	Professor, Department of Pharmacology, IPGME&R

* Inducted into Committee on 04.07.2015 in place of former director Prof. Pradip Kumar Mitra.

Avijit Hazra 10/12/2015

Dr. Avijit Hazra – Member Secretary
IPGME&R Research Oversight Committee

Jayita Basu. 05/11/19.

Member Secretary
Institutional Ethics Committee
Institute of Postgraduate Medical
Education & Research [IPGME&R]
Kolkata-700020